***In Vitro* Properties of Leukocyte Dialysates Containing Transfer Factor**

(delayed hypersensitivity/cellular immunity/lymphocyte transformation)

MICHAEL S. ASCHER*, WILLIAM J. SCHNEIDER, FRED T. VALENTINE, AND H. SHERWOOD LAWRENCE

Infectious Disease and Immunology Division, Department of Medicine, New York University School of Medicine, New York, N.Y. 10016

Contributed by H. Sherwood Lawrence, September 13, 1973

ABSTRACT The chief impediment to the precise biochemical identification of transfer factor and its mechanism of action has been the lack of a reproducible *in vitro* assay. We now report on a method by which dialysates containing transfer factor of proven *in vivo* potency can convert nonimmune lymphocytes to immune responsiveness *in vitro*, as reflected by antigen-triggered lymphocyte proliferation. However, "water-dialyzed transfer factor" (TF_D) prepared for *in vivo* use by the conventional method of dialysis against large volumes of water exhibits diminished activity *in vitro* and is frequently toxic to lymphocyte cultures. This problem can be avoided by dialysis of transfer factor into tissue culture medium. When this precaution is taken, such "media-dialyzed transfer factor" (TF_{DM}) causes nonimmune lymphocytes to respond to antigen by an increment of thymidine incorporation that ranges from 2 to 25 times that of such cells cultured with antigen alone. This response is generally observed only in the presence of those antigens to which the TF_{DM} donor expresses delayed cutaneous reactivity and is distinguishable from nonspecific adjuvant effects.

Cellular immunity has been implicated in recovery from various bacterial, viral, and fungal infections and in the rejection of transplants and surveillance against malignant tissues (1). Following the transfer of cellular immunity in the guinea pig by Landsteiner and Chase (2) the transfer of DTH in man was accomplished using viable blood leukocytes or leukocyte extracts (3, 4). The active material in leukocytes or leukocyte extracts is a soluble, dialyzable, nonantigenic moiety of peptide-nucleotide composition that is less than 10,000 molecular weight with *in vivo* potency equivalent to that of the parent leukocyte preparations (5). This material has been designated dialyzable transfer factor (TF_D) and its nature and properties have been extensively confirmed (6-8). Numerous recent clinical applications of TF_D in immunotherapy have led to dramatic clinical and immunologic improvement in congenital immunodeficiency states (9, 33-35), and in infectious diseases caused by viruses (10), fungi (11-14), or mycobacteria (15, 16). Transfer factor therapy has also resulted in immunologic improvement and occasional clinical improvement in some patients with metastatic cancer (17-21). The precise mechanism by which this small molecule confers specific immune responsiveness on the recipient and his circulating lymphocytes is unknown since characterization of the molecular species and mechanism of action of TF has been impeded by the lack of a suitable animal model or an *in vitro* assay for its

biologic activity. TF_D is not antigenic and is separated from histocompatibility antigens (22, 23), properties that allow it to be cultured with nonimmune lymphocytes as an *in vitro* assay system. In this system lymphocytes from nonimmune, skin-test negative individuals are cultured with TF and examined for the acquisition of the ability to be stimulated by antigen as measured by transformation and increased thymidine incorporation. Preliminary results on the development of this *in vitro* assay have been reported from this laboratory (24, 25) as well as others (26, 27) but were limited to sporadic observations since technical problems hampered further exploitation of this system. In this report we outline observations to show a more consistent amplification of lymphocyte reactivity obtained when cultured with TF preparations that are dialyzed directly into tissue culture medium (TF_{DM})

MATERIALS AND METHODS

Selection of Donors and Recipients of Transfer Factor. The peripheral blood leukocytes used in these *in vitro* studies were obtained from normal young adult volunteers. The donors of leukocytes were selected for either marked delayed cutaneous reactivity (20 × 20 mm induration) or complete absence of reactivity to the antigen under study [e.g., purified protein derivative (PPD): 0.1 μg; diphtheria toxoid: 0.008 flocculation unit (Lf); streptokinase-streptodornase (SK-SD): 40-10 units; or 0.1 ml of coccidioidin]. Nonimmune donors of lymphocytes to be cultured with TF exhibited no skin-test reactions when tested with either 5 μg of PPD or equivalent screening concentrations of the other antigens. In addition to delayed cutaneous reactivity *in vivo*, the responses of prospective donors and recipients of TF were also assessed in the *in vitro* lymphocyte transformation test (28). In many experiments only the lymphocyte transformation test was performed and skin-testing was not done. Two of the donors (HUC, JOS—see Table 2) of TF_{DM} for these *in vitro* experiments were selected because of 20 years' experience documenting the *in vivo* potency of their TF preparations (4, 22, 25).

Preparation of Dialyzable Transfer Factor. Venous blood is obtained with preservative-free heparin (10% v/v of a 2 mg/ml of solution in saline—Mann Research) and dextran (Pharmachem 186,000 molecular weight or Sigma 252,000 molecular weight—10% v/v of a 6% solution in saline) and allowed to sediment at 37° in tissue culture tubes for 30-40 min. The plasma white cell supernatant is removed and centrifuged at 200 × g at 4° for 10 min. The white cell pellet is suspended in sterile water (2 ml per 10⁸ lymphocytes). Two-milliliter aliquots are placed in sterile Pyrex tubes, frozen in dry ice and stored at -20°. They are withdrawn as needed for further processing in separate batches. To each tube of cells 100 μg of twice-recrystallized pancreatic DNase (Worthing-

Abbreviations: dpm, disintegrations per minute; PHA, phytohemagglutinin; PPD, purified protein derivative (of tuberculin); SK-SD, streptokinase-streptodornase; TF, transfer factor; TF_D, water-dialyzed transfer factor; TF_{DM}, media-dialyzed transfer factor; Lf, flocculation unit.

* Current address: USAMRIID, Fort Detrick, Frederick, Md. 21701.

ton—Code DCL11S) is added and the cells frozen in dry ice-acetone and thawed at 25° for 10 cycles. The lysate is pipetted into a Visking dialysis tubing (8-mm) which has been boiled twice in water. The tubing is tied using sterile gloves and double knots placed at each end. The bag is placed in the dialysis fluid with the ends and knots above the surface of the liquid. A small boiled magnetic stirrer is added.

Dialysis of TF_D . The material is prepared as if for *in vivo* use: The leukocyte lysate is dialyzed for 18 hr at 4° against 200 volumes (400 ml) of USP sterile distilled water for injection (5, 29). The dialysate is lyophilized and the powdered lyophilizate is reconstituted in water (1 ml for each 3×10^7 lymphocytes), passed through a washed 0.45 μ m Millipore filter and frozen at -20° until use.

Dialysis of TF_{DM} . To prepare the material for *in vitro* use, dialysis of the 2-ml leukocyte lysate containing 10^8 lymphocytes is carried out against minimal essential medium for suspension cultures (MEM-S—Microbiological Assocs.) for 18 hr with 1 ml of medium for every 6×10^6 lymphocytes in the bag (30). The ratio of inside to outside volume is about 1 to 10. The dialysate medium containing the TF_{DM} is filtered and stored at 4° until use. The termination of dialysis at the end of 18 hr is largely a matter of convenience since an additional 18-hr dialysis will yield a small additional increment of active material.

Preparation of Lymphocyte Cultures. The leukocytes for culture are separated in a manner identical to that employed in the preparation of transfer factor detailed above. The plasma is removed from the white cells, clarified of platelets by centrifugation, and added to MEM-S at a concentration of 20%. The medium contains added glutamine and ampicillin at concentrations of 2 mM and 100 μ g/ml, respectively. Three milliliters of the cell suspension containing 1.5 to 2×10^6 lymphocytes is placed in each culture tube and 1 ml of either TF_{DM} or medium is added for a total of 4 ml, resulting in a final plasma concentration of 15%. The cells are incubated at 37° in an atmosphere of 5% CO_2 for 6 days at which time 0.1 ml of [^{14}C]thymidine is added. The cells are harvested on the seventh day and uptake of thymidine is determined by the method previously described from this laboratory (31). The choice of 7 days as the length of culture is based on time-course experiments that demonstrate maximal differences caused by TF_{DM} are detected that day.

Preparation of Antigens for In Vitro Use. (1) Tuberculin PPD (Merck, Sharpe and Dohme—second strength, or Connaught-preservative-free, courtesy of Dr. S. Landi) is added at 4 μ g per tube in 0.1 ml of MEM-S. Both of these preparations have yielded equivalent results in this *in vitro* system. (2) Streptokinase-streptodornase (SK-SD—Varidase—Lederle dialyzed versus saline to remove preservative) is added as 40 units of SK—10 units of SD per tube in 0.1 ml of MEM-S. (3) Purified diphtheria toxoid (courtesy of Dr. G. Grady—Massachusetts Department of Health—prepared free of preservative for *in vitro* use) is added as 0.23 Lf per tube in 0.1 ml of saline. (4) Coccidioidin (courtesy of Dr. D. Pappagianis—dialyzed to remove merthiolate) is added as 0.1 ml per tube. The concentrations of antigens listed above were found to be optimal for the stimulation of natively sensitive lymphocytes and were the standard concentrations used in all these experiments.

RESULTS

We have measured thymidine incorporation stimulated by PPD in lymphocytes obtained from individuals with negative

skin-tests and those with positive skin-tests. Lymphocytes obtained from 110 PPD-negative individuals when cultured without PPD incorporated 231 disintegrations per minute (dpm) per tube (193–271; geometric mean and 95% confidence limits) versus 5177 dpm (4373–6129) when cultured with 1 μ g/ml of PPD. In contrast lymphocytes obtained from 20 PPD-positive individuals incorporated 206 dpm (161–266) when cultured without PPD versus 30,204 dpm (24,024–37,687) when cultured with 1 μ g/ml of PPD.

The magnitude of this difference allows us to screen individuals without exposure to skin-testing. PPD regularly causes this background amount of stimulation of lymphocytes obtained from most individuals negative to a 5- μ g skin-test with PPD. An assessment of the possible contribution of such background stimulation characteristic of this antigen to the observed effects of TF_{DM} *in vitro* will be presented below (Fig. 5).

Effects of TF Alone on Immune and Nonimmune Lymphocytes. In an attempt to evaluate whether TF functions as superantigen *in vitro*, we have added tuberculin-positive TF alone in large quantities (e.g., that prepared from 10^8 lymphocytes per tube) to lymphocytes obtained from individuals with exquisite sensitivity to tuberculin and have observed no stimulation of transformation. This *in vitro* assay will detect as little as 1 ng of antigen in the tuberculin system (28). We have also added TF alone to nonimmune lymphocytes and have observed no significant stimulation or transformation in over 100 experiments. Thus, within the limits of discrimination of this assay system for the detection of antigen, TF does not behave as an antigen. This property allows TF to be studied for its role in preparing lymphocytes to respond to antigen (also see TF_{DM} controls in Figs. 1–6).

Effects of TF Plus Antigen on Nonimmune Lymphocytes. (A) *Employing "water-dialyzed transfer factor" (TF_D).* When tuberculin-positive TF_D prepared for *in vivo* use, as described above, was added to lymphocytes in the presence of PPD, enhancement of thymidine incorporation occurred. In extensive studies with this preparation, however, thymidine incorporation in cultures with TF_D plus antigen was usually only 1.5–3 times that in cultures with antigen alone and often not statistically significant. Moreover, such limited increments of lymphocyte stimulation were seen in only 23% of attempts with PPD as antigen. Because the addition of TF_D , either alone or in the presence of antigen, resulted frequently in slight depression of lymphocyte transformation we attempted to detect an inhibitor in the TF_D preparation. Since TF_D activity *in vivo* can be separated from inactive components on Sephadex G-25, we applied this technique to two raw dialysates that were not active *in vitro* and retested the separate fractions. In each case the excluded fraction contained inhibitory material and the retarded fraction exhibited minimal activity. This clue led us to suspect the introduction of a material toxic for lymphocyte cultures consequent to the method of preparation of TF_D .

(B) *Employing "media-dialyzed transfer factor" (TF_{DM}).* In an attempt to diminish the toxicity and increase the activity of TF preparations, lyophilization was eliminated by dialysis of the leukocyte lysate directly into a small volume of physiologic tissue culture medium (TF_{DM}). Fig. 1 outlines the results of the addition of TF_{DM} to nonimmune cells in culture and compares it to the addition of a standard "water-dialyzed" TF_D made in parallel from an equal aliquot of the same white cell lysate. It may be seen that there is no stimulation of

FIG. 1. Transfer factor *in vitro*: media-dialyzed TF_{DM} is active compared to water-dialyzed TF_D. Each aliquot of TF = 6×10^6 lymphocytes. In this and all figures bars represent means and dots (·) individual replicate points. Also, numbers on the ordinate were obtained by multiplying the experimental values by the given factor, 10^{-3} .

thymidine uptake in cultures in which TF_{DM} alone is present. TF_D prepared from 6×10^6 lymphocytes plus antigen also gave no increase. In contrast TF_{DM} from 6×10^6 lymphocytes plus antigen resulted in a 30-fold increase in thymidine incorporation over antigen alone.

Since the intensity and duration of the effects of TF_D *in vivo* are a function of the dose employed (22), we studied the effects of increasing the quantity of TF_{DM} added to lymphocyte cultures *in vitro*. In Fig. 2 we illustrate the initial segment of a dose-response curve of nonimmune lymphocytes to increasing amounts of tuberculin-positive TF_{DM} in the presence of constant amounts of antigen. It may be noted that the magnitude of the lymphocyte response increases progressively as more TF_{DM} is added. We have also observed a comparable dose-response effect of toxoid-positive TF_{DM} in the presence of diphtheria toxoid. The overall response of lymphocytes to TF_{DM} plus antigen in these experiments appears to be a function of the amount of TF_{DM} added up to an optimum dose.

We have secured additional data not tabulated here to show that doses of TF_{DM} in excess of 20×10^6 lymphocyte equivalents per tube can result in a depressive effect on recipient lymphocyte cultures. Since TF_{DM} from 6×10^6 lymphocyte equivalents will give statistically significant differences between control and experimental cultures, we have chosen

FIG. 2. Dose response to tuberculin-positive TF_{DM} in the presence of a constant concentration of tuberculin PPD. Each ml of TF = 6×10^6 lymphocytes.

this dose range for most of the experiments presented here.

In Table 1 we list the results of a series of experiments where both the concentration of TF_{DM} and the concentration of the antigen are held constant. The lymphocyte responses in 14 such experiments are ranked according to the ratio of PPD plus TF_{DM} over PPD alone. Because of the level of background stimulation caused by PPD alone, the ratios obtained using this antigen are usually less than those obtained using other antigens that cause insignificant amounts of background stimulation (e.g., diphtheria toxoid, SK-SD). However, the differences that are obtained and illustrated here are significant ($P < 0.05$). For these and other reasons (e.g., possible stimulation of B-cells by PPD; crossreactivity with anonymous mycobacterial antigens) we do not consider tuberculin-PPD an ideal antigen for such *in vitro* studies. Nevertheless, despite its disadvantages, positive results using PPD as antigen are obtained in 50–60% of attempts when nonimmune lymphocytes are incubated with TF_{DM} from donors who are exquisitely sensitive to tuberculin.

Evaluation of Specific Activities of TF. (A) Transfer factor usually conveys skin and lymphocyte reactivity *in vivo* concordant with the pattern and intensity of the sensitivities expressed by the TF donor (9, 13, 25, 26). To test for specificity of TF_{DM} *in vitro* we utilized a transfer factor donor, JOS (markedly reactive to PPD and SK-SD but not to diphtheria

TABLE 1. Tuberculin stimulation of non-immune lymphocytes: range of responses to constant amounts of TF_{DM} and PPD

Immune donor	Nonimmune recipient	No addition	PPD 4 μg	TF _{DM} *	TF _{DM} * + PPD 4 μg	Ratio†
JOS	MA	1834 ± 304	7624 ± 3581		26886 ± 3808	3.53
JC	MA	204 ± 91	1111 ± 440	298 ± 110	3601 ± 1602	3.24
JOS	JB	268 ± 64	2697 ± 559	484 ± 191	8534 ± 683	3.17
JC	BA	276 ± 45	3495 ± 1504	411 ± 18	10451 ± 3008	2.99
JK	PB	138 ± 22	1688 ± 485		4593 ± 495	2.94
DK	JM	401 ± 22	4688 ± 236	392 ± 33	13328 ± 2157	2.84
JS	AL	538 ± 198	8645 ± 969		23634 ± 3597	2.73
WS	MA	1379 ± 100	16633 ± 5143		42850 ± 5944	2.58
JC	AL	960 ± 315	11612 ± 3249		29269 ± 2618	2.52
JOS	PB	106 ± 1	4054 ± 210		8938 ± 2056	2.21
JOS	PO	202 ± 71	7099 ± 1994		14927 ± 4329	2.10
HUC	MA	214 ± 33	3041 ± 659		6534 ± 1256	2.09
JOS	PO	360 ± 68	4901 ± 1326		9469 ± 2021	1.93
JOS	BA	1806 ± 354	16834 ± 4831	2407 ± 701	32224 ± 2652	1.92

* TF_{DM} prepared from 6×10^6 lymphocytes.

† Ratio of (TF_{DM} + PPD)/(PPD alone).

‡ Mean and SD of triplicate or quadruplicate tubes in dPM of [¹⁴C]thymidine incorporated.

FIG. 3. Concordance of specificity of TF_{DM} *in vitro* with donor's cutaneous sensitivity and ability to transfer *in vivo*. Donor is PPD and SK-SD positive, toxoid negative. Each aliquot of TF = 6×10^6 lymphocytes.

toxoid), who has previously been shown to transfer regularly skin reactivity *in vivo* to PPD and SK-SD but not to diphtheria toxoid (4, 17, 24, 25). TF_{DM} was prepared from this donor and added to lymphocytes obtained from an individual without skin reactivity to PPD, SK-SD, or diphtheria toxoid. It may be seen in Fig. 3 that the enhanced stimulation resulting from exposure of nonimmune lymphocytes to TF_{DM} occurred only in the presence of those antigens to which the TF donor was sensitive.

(B) TF_{DM} was prepared from an individual without geographic exposure to *Coccidioides* who had never been skin-tested with coccidioidin and who was judged nonreactive to antigen solely by means of the lymphocyte transformation test. TF_{DM} was also prepared in parallel from a donor with marked cutaneous reactivity to coccidioidin. Both the coccidioidin-positive and the coccidioidin-negative TF_{DM} aliquots were prepared from 6×10^6 lymphocytes and added to lymphocytes obtained from another coccidioidin-negative individual. As shown in Fig. 4 it was observed in a single experiment that only the coccidioidin-positive TF_{DM} enhanced the response of coccidioidin-negative cells to antigen. Coccidioidin-negative TF_{DM} at dose ranges equivalent to 3×10^6 and 9×10^6 lymphocytes also failed to cause an enhanced response to coccidioidin. Thus TF_{DM} from negative reactors in these two types of experiment failed to activate lymphocytes to respond to the corresponding antigen. Nevertheless, many more experiments using a variety of negative donors will be required before any firm conclusion concerning the specificity of TF_{DM} *in vitro* can be reached.

Evaluation of Nonspecific Enhancement by TF_{DM} In Vitro.

(A) If the above effects were merely a consequence of adjuvant activity, then one should expect that TF_{DM} would more

FIG. 4. Specificity of TF_{DM} *in vitro*: coccidioidin-positive (cocci-pos) TF_{DM} enhances the response to coccidioidin. Coccidioidin-negative (cocci-neg) TF_{DM} is ineffective. Each aliquot of TF = 6×10^6 lymphocytes.

FIG. 5. Response to TF_{DM} and its magnitude is not dependent on background response to antigen alone. Each aliquot of TF = 6×10^6 lymphocytes.

readily enhance lymphocyte stimulation to antigens to which the recipient already expresses a small background response than to antigens to which recipient lymphocytes are completely unresponsive *in vitro*. In the experiment illustrated in Fig. 5 we test this possibility using lymphocytes from an individual who exhibits moderate *in vitro* stimulation to PPD but no stimulation to SK-SD. It may be seen that the magnitude of the response of the cells cultured with a TF_{DM} positive for both PPD and SK-SD is greater in the presence of SK-SD than in the presence of PPD. These results would suggest that the status of the donor's sensitivity plays a major role in determining the response to TF_{DM} and would tend to exclude an adjuvant effect elevating latent responses of recipient cells.

(B) If TF_{DM} were to amplify lymphocyte transformation nonspecifically, then this effect might be detected on lymphocytes cultured with phytohemagglutinin, PHA. In Fig. 6, we added TF_{DM} from a tuberculin-positive donor to tuberculin-negative lymphocytes that were stimulated by sub-optimal doses of PHA. It may be seen that the TF_{DM} did not enhance the stimulation of lymphocytes by PHA, whereas the response to PPD is enhanced by the TF_{DM}.

(C) Other materials like synthetic polynucleotides cause increased lymphocyte responses to antigen but this effect can only be demonstrated with suboptimal antigen concentrations (32). To minimize the possible contribution of this type of nonspecific stimulation to the results observed, only optimal doses of antigen were used in all experiments assessing activity of TF_{DM}.

(D) To evaluate the possible contribution of endotoxin, lipopolysaccharide from *Escherichia coli* and/or *Salmonella* was added in various concentrations to nonimmune lymphocytes in the presence or absence of PPD. Endotoxin alone in

FIG. 6. TF_{DM} fails to enhance response of lymphocytes to sub-optimal doses of PHA. Each aliquot of TF = 6×10^6 lymphocytes.

0.1- μ g, 1.0- μ g, 10- μ g, and 100- μ g quantities resulted in lymphocyte stimulation ranging from 4,068 dpm to 14,324 dpm. In contrast TF_{DM} does not by itself cause lymphocyte stimulation.

Endotoxin in the above amounts did not enhance the response of nonimmune lymphocytes to 4 μ g of PPD (20,264 dpm) whereas TF_{DM} prepared from 6×10^6 lymphocytes of a tuberculin-sensitive donor increased the response of the same recipient's cells to 4 μ g of PPD up to 67,547 dpm in the same experiment.

DISCUSSION

The cumulative data from earlier as well as current *in vivo* experience suggest that TF acts operationally as though it either uncovers or causes the appearance of new receptor sites on the recipients' blood lymphocytes resulting in the generation of a new clone of specific antigen-reactive cells, or that it activates and increases a clone of cells already programmed to respond to a given antigen (9, 22, 36). The data reported here indicate that reproducible results can be achieved using TF_{DM} to convert nonimmune lymphocytes to an antigen-reactive state *in vitro*. The results secured include a selection of TF_{DM} specificities corresponding to a variety of antigen systems. These preliminary studies of TF_{DM} *in vitro* have revealed the activity to be specific in one type of test, where the same donor is sensitive to two out of three antigens used and in another type of test comparing the response of coccidioidin-negative lymphocytes to either coccidioidin-positive or coccidioidin-negative TF_{DM}. However, more detailed evaluation of this question is necessary before any firm conclusions can be reached. Any nonspecific adjuvant effects that may exist have not been detected under the conditions of these experiments. This is not to say, however, that nonspecific consequences of specific lymphocyte activation may not be detectable in other systems or when end-points other than that of lymphocyte transformation are employed as TF_{DM} assays (e.g., macrophage activation; T-B cell facilitation; mediator production; target cell destruction).

It must be emphasized that the need for the special preparation of TF_{DM} is a maneuver designed to meet the fastidious requirements of the lymphocyte culture system not encountered *in vivo*, because of the closed nature of the *in vitro* system where minute losses of activity or the introduction of inhibitors is greatly magnified.

Whether the activity we have described *in vitro* is an exclusive property of the same active moiety of leukocyte dialysate that functions *in vivo* or an expression of an additional immunologic phenomenon remains to be established. In any event the assay represents a crude beginning subject to additional modifications and improvement. Nevertheless, it gives promise of further refinement and adaptation to the preliminary assessment of steps designed to purify and concentrate TF, to determine its biochemical nature and to study its mechanism of action preparatory to parallel *in vivo* evaluation.

Preliminary reports of this work were presented in part at the NIH Workshop of "Basic Properties and Clinical Applications of Transfer Factor," Tucson, Arizona, February 16, 1973 and at the 57th annual meeting of the Federation of American Societies for Experimental Biology (30).

We thank Ms. Peggy Orenstein-Reyhani for her excellent technical assistance and all of our blood donors for their cooperation in making these studies possible. These studies were supported in part by the United States Public Health Service, Research Grant

AI 01254-17 and Training Grant AI 00005-14, and in part by the Armed Forces Epidemiological Board. M.S.A. and W.J.S. were USPHS Trainees and F.T.V. holds a Career Scientist Award, Health Research Council, New York City (I-637).

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