

REQUIREMENT OF PRECOMMITTED CELLS AS TARGETS FOR THE AUGMENTATION OF LYMPHOCYTE PROLIFERATION BY LEUKOCYTE DIALYSATES*

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While extensive studies in humans have documented the biological and immunological properties of transfer factor in vivo, it is apparent that an in vitro assay system is required for further biochemical purification and characterization, as well as for analysis of its mechanism of action (1, 2). The term transfer factor (TF)¹ was originally introduced to designate those components in leukocyte extracts responsible for the in vivo transfer of delayed-type hypersensitivity in man (3). The term is a convenient operational description and not a biochemical entity, and it is employed with the appreciation that more than one factor may be involved in the effects observed. It is understood that dialysates of leukocytes are complex mixtures of small molecules which in addition to immunologically specific components of TF, also contain other low molecular weight materials with potential biological activity. An assessment of the relative contributions of such specific and nonspecific components to the in vivo and in vitro activities of leukocyte dialysates is an obligatory prelude to further purification and characterization of TF and to the design of a meaningful in vitro assay of its activity.

We have recently described preliminary studies assessing lymphocyte blastogenesis as a potential assay for TF activity (4, 5). In this system it was demonstrated (5) that when prepared in tissue culture medium, leukocyte dialysates containing TF augmented antigen-triggered proliferation of human lymphocytes in vitro. Preliminary experiments designed to assess the immunological specificity and underlying mechanism(s) of the phenomenon were also reported. These observations have been subsequently confirmed and extended to show that the augmentation caused by antigen in the presence of leukocyte dialysate was greater on those recipient lymphocytes which had a higher baseline proliferative response to antigen alone (6-9).

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¹ *Abbreviations used in this paper:* Ag, antigen; [¹⁴C]TdR, ¹⁴C-labeled thymidine; D.Tox., diphtheria toxoid; Δcpm, the arithmetic difference in cpm between cultures containing antigen plus leukocyte dialysate and cultures containing just antigen; [³H]TdR; tritiated thymidine; LF, flocculation unit; ln, natural log; MEMS, minimal essential media for suspension cultures; PPD, purified protein derivative of tuberculin; SK-SD, streptokinase-streptodornase; TF, transfer factor; TU, tuberculin units.

To facilitate the analysis of the action of leukocyte dialysate we have developed a microculture adaptation of the macrosystem reported earlier (5). With this technique it has become possible to apply multiple dialysates to the recipient lymphocytes of a single individual and conversely to assay a single dialysate preparation on the lymphocytes of multiple recipients. This adaptation facilitates the determination of the relative contributions of the dialysate and cultured lymphocytes to the augmentation observed.

In this report we present detailed evidence to show that the augmentation of lymphocyte proliferation produced by leukocyte dialysates plus antigen: (a) does not occur when precommitted, antigen-specific lymphocytes are deleted from the recipient population by the "hot-pulse" technique of Dutton and Mishell (10); and (b) appears to be due mainly to a nonspecific adjuvant effect of leukocyte dialysates, the magnitude of which is determined by the immune reactivity of recipient lymphocytes, rather than that of the leukocyte dialysate donor.

Materials and Methods

Selection of Donors and Recipients of Leukocyte Dialysates. All donors were normal adult human volunteers with a normal leukocyte and differential count at the time of venipuncture. The donors for dialysate preparations as well as those employed for recipient leukocyte cultures were selected to cover a broad spectrum of delayed cutaneous reactivity from complete absence of reactivity to exquisite hypersensitivity to the antigens under study. Skin tests were performed with purified protein derivative of tuberculin (PPD): 5 tuberculin units (TU) or 250 TU; or diphtheria toxoid (D.Tox.): 0.008 flocculation unit (Lf). Skin tests of 10 mm or more induration at 48 h were considered positive. In addition to delayed cutaneous reactivity *in vivo*, the responses of prospective donors and recipients of dialysate were also assessed by a microadaptation of the lymphocyte transformation test (11).

Preparation of Leukocyte Dialysates. Each 10 ml of venous blood were mixed with 1.0 ml of preservative-free, sodium heparin (Schwarz/Mann Div., Beton, Dickinson & Co., Orangeburg, N. Y.; 100,000 U/g, 2 mg/ml) and 1.0 ml of 6% dextran (Sigma Chemical Co., St. Louis, Mo.; average mol wt, 250,000) in saline, and the erythrocytes were allowed to sediment for a half hour at 37°C in glass tubes (16 × 125 mm). The leukocyte-rich plasma was removed and total and differential cell counts were performed. This plasma was centrifuged at 300 *g* for 15 min at 5°C. The cell pellet was then suspended in 1 ml of sterile double-distilled water and immediately frozen in a dry-ice-acetone bath. These frozen pellets were stored at -20°C or below. Pancreatic DNase I (Worthington Biochemical Corp., Freehold, N. J.; twice recrystallized), 0.1 mg, was added to the lysate which was then subjected to 10 cycles of freezing and thawing between a dry-ice-acetone bath and a 25°C water bath. The lysate was then dialyzed against minimal essential media used for suspension cultures (MEMS) (Microbiological Associates, Bethesda, Md.). The dialysis tubing (Arthur H. Thomas Co., Philadelphia, Pa.; 1/4 inch diameter, 0.002 inch wall thickness, initial average pore size 48 Å) was washed twice and boiled twice in sterile pyrogen-free H₂O or saline. The dialysate volume was adjusted to yield a leukocyte extract of 1.0 × 10⁷ mononuclear cell equivalents/ml of dialysate. After an overnight dialysis, the dialysate was filtered (Millipore Corp., Bedford, Mass.; Millex filter, 0.45 μm) and stored at 4°C until use. The temperature and pH were adjusted before the addition of experimental leukocyte suspensions by placing the dialysate in a 37°C 5% CO₂ incubator.

Deletion of Clones of Antigen-Reactive Cells with [³H]Thymidine of High Specific Activity. Venous blood was sedimented at 37°C for a half hour with heparin and dextran as for dialysate preparations. A differential count was performed and the leukocyte-rich plasma was centrifuged at 300 *g* for 15 min at room temperature. The cell pellet was resuspended in MEMS supplemented with 1% 200 mM glutamine (Microbiological Associates) and 50 μg/ml gentamicin (Schering Diagnostics Div., Schering Corp., Port Reading, N. J.) while the supernatant plasma was clarified by centrifugation at 900 *g* for 20 min.

A modification of the method of Hirschberg and Thorsby (12) was used. 2- or 4-ml cultures were

established in glass culture tubes (16×125 mm) containing 1.0×10^6 mononuclear cells/ml of media with 15% autologous plasma. Antigens were added to duplicate or triplicate cultures at the following concentrations: PPD (Connaught Laboratories, Toronto, Canada; PPD-CT 68, preservative free, lot A-11)² 2.5 μ g/ml; D.Tox. (Massachusetts Public Health Biologic Laboratory, Boston, Mass.; preservative free)³ 8.25 Lf/ml; or streptokinase-streptodornase (SK-SD) (Lederle Laboratories, Pearl River, N. Y.; Varidase, dialyzed to remove preservative) 50 SK and 12.5 SD U/ml. Antigen concentrations were chosen to stimulate responsive cells optimally. These antigen-stimulated cultures and parallel cultures without antigen were incubated at 37°C in a humidified 5% CO₂ incubator. At 24 and again at 48 h both test and control cultures received 2.5 μ Ci/ml of tritiated thymidine [³H]TdR (New England Nuclear, Boston, Mass.; sp act, 20 Ci/mmol) to inhibit proliferating cells.

At 72 h the extracellular [³H]TdR was removed by two washes with MEMS, 5 ml each. Replicate tubes were pooled and the cells resuspended at approximately 1.5×10^6 mononuclear cells/ml in MEMS supplemented with 30% autologous plasma and double the above concentrations of glutamine and gentamicin.

Microtechnique for Leukocyte Culture with Antigen and Leukocyte Dialysate. The cell population thus prepared and depleted of cells responding by proliferation to a specific antigen were then cultured with both the specific and unrelated antigens using a microtechnique. Each group was assayed in triplicate for [¹⁴C]thymidine incorporation.

0.1 ml of the [³H]TdR pulsed cell suspensions were added to an equal volume of either MEMS or leukocyte dialysate in sterile round-bottomed microplates (Linbro Chemical Co., New Haven, Conn.; no. IS-MRC-96TC). To these secondary cultures PPD, D.Tox., or SK-SD was added at the same concentrations as had been used in the primary cultures. Blastogenesis in cultures without additional antigen was also measured.

Microcultures were incubated at 37°C in a humidified 5% CO₂ incubator. 6 days later thymidine incorporation was measured by the addition of 0.03 μ Ci of [¹⁴C]thymidine ([¹⁴C]TdR) (New England Nuclear; sp act, approximately 50 μ Ci/mmol) to each well. Distilled water lysates or TCA precipitates were harvested 8–10 h later on glass fiber filters and counted in a liquid scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.; LS 150) equipped with an automatic quench control, which minimized any ³H spillover into the ¹⁴C window. This machine also compensated for quenching, allowing all samples to be counted at a constant efficiency. All results are therefore reported as counts per minute of [¹⁴C]TdR incorporated during the last 8–10 h of culture.

Analysis of Data. Data were studied in the form of raw counts per minute, natural log (ln) counts per minute, and stimulation ratios (as described in the Results). Use of the ln counts per minute normalized the error (13) and was therefore used to perform statistical analyses. Analyses of variance, regression, and covariance were performed on a PDP-8 computer according to standard methods (14).

Results

Spectrum of Donor Reactivity. The lymphocytes of the individuals who served as donors for these experiments covered a broad range of reactivity in vitro for the three antigens employed. As shown in Table I, stimulation ratios (counts per minute with antigen/counts per minute without antigen) ranged from 1 to 102 for PPD, 1 to 22 for D.Tox., and 1 to 47 for SK-SD. The group of six individuals that served as donors of dialysate and the eight individuals whose lymphocytes served as recipients, each covered a comparable broad range of reactivity. Stimulation ratios of 9 or greater corresponded to at least 10 mm induration with PPD or D.Tox.

Lack of Mitogenic Effect by Leukocyte Dialysates on Unstimulated Cultures. In this microsystem the addition of dialysate alone did not stimulate

² Generous gift of Dr. S. Landy.

³ Generous gift of Dr. G. Grady.

TABLE I
In Vitro Reactivity of the Donors of Leukocyte Dialysates and Recipient Lymphocyte Cultures

| | cpm Thymidine incorporation | | | |
|----------------------------------|-----------------------------|---------------|--------------|--------------|
| | No anti- gen | PPD | D.Tox. | SK-SD |
| A. Dialysate donors | | | | |
| HuC | 34 | 2,346 (69.0)* | 40 (1.2) | 634 (18.6) |
| W. S. | 18 | 1,839 (102.2) | 77 (4.3) | 240 (13.3) |
| V. L. | 122 | 116 (0.9) | 76 (0.6) | 126 (1.0) |
| B. S. | 17 | 825 (48.5) | 306 (18.0) | 338 (19.9) |
| R. R. | 74 | 3,222 (43.5) | 1,613 (21.8) | 1,856 (25.1) |
| C. M. | 87 | 113 (1.3) | 290 (3.3) | 111 (1.3) |
| B. Recipient lymphocyte cultures | | | | |
| B. A. | 31 | 99 (3.2) | — | 235 (7.6) |
| J. C. | 51 | 3,264 (64.0) | — | 2,372 (46.5) |
| W. S. | 42 | 2,466 (58.7) | 145 (3.5) | 266 (6.3) |
| F. V. | 36 | 39 (1.1) | 468 (13.0) | 236 (6.6) |
| D. V. P. | 52 | 483 (9.3) | 54 (1.0) | 171 (3.3) |
| A. S. | 23 | 1,678 (73.0) | 47 (2.0) | 37 (1.6) |
| J. Ca. | 33 | 27 (0.8) | 32 (1.0) | 259 (7.8) |
| L. G. | 65 | 836 (12.9) | 303 (4.7) | 186 (2.9) |

* Geometric mean of the cpm of [¹⁴C]TdR incorporated in triplicate cultures. Numbers in parentheses are stimulation ratios (cpm with antigen divided by cpm without antigen).

normal lymphocytes. In eight consecutive experiments, the triplicate sets of cultures in the absence of antigen had a geometric mean of 37 cpm (range 23–65). In the presence of dialysate alone, these sets had a geometric mean of 34 cpm (range 21–94), a difference that is not statistically significant. Moreover, dialysate from sensitive donors did not behave as an antigen in cultures of leukocytes obtained from donors with exquisite cutaneous reactivity to the same antigen. These cultures are capable of response to nanogram quantities of antigen (15).

Augmenting Effect of Leukocyte Dialysates on Antigen-Stimulated Cultures. Dialysates usually enhanced the incorporation of thymidine by antigen-stimulated lymphocytes. Pooling all data from nonsensitive and sensitive individuals, in 46 different combinations of dialysate and recipient cells, the mean thymidine incorporation was increased 2.23 ± 0.26 SE-fold over that seen with antigen (Ag) alone in cultures without specific clonal deletions.⁴ Fig. 1 shows the cumulative frequency distribution of the augmentation ratios (cpm [Ag + dialysate]/cpm[Ag]). The median ratio was only 1.3 and in approximately one-third of the trials the ratio was less than one. The augmentation ratio remained constant with increasing reactivity of the recipient cells to antigen alone (correlation coefficient, 0.04). However, as we had initially observed [see Table I in Ascher et al. (5)], the arithmetic difference between the counts per minute incorporated by antigen-stimulated lymphocytes in culture with and without

⁴ One of nine dialysates, prepared for these studies, was toxic and has not been included in the data because it consistently and nonspecifically depressed the background level of response of cells stimulated by antigen.

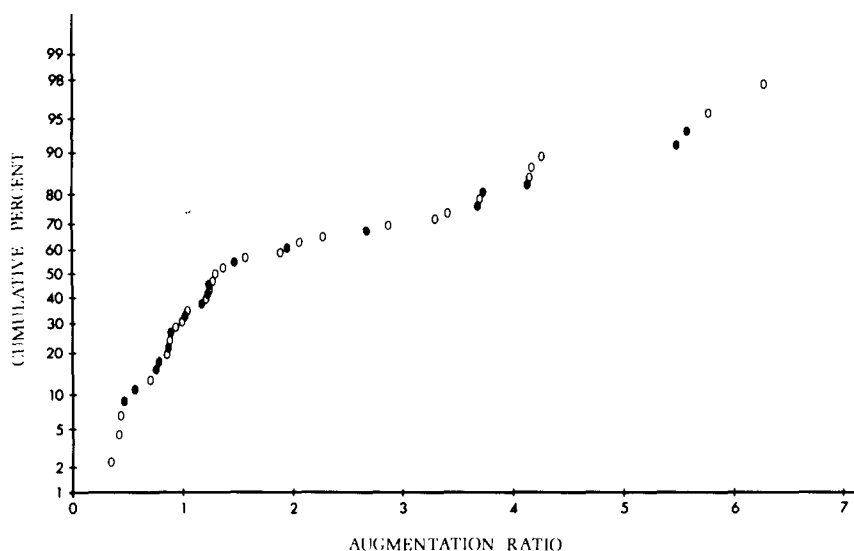


FIG. 1. Cumulative frequency distribution of augmentation ratios obtained when 46 combinations of donor cells and leukocyte dialysates were cultured with antigen. Each point represents triplicate cultures. Closed circles represent dialysate from nonsensitive donors and open circles from sensitive donors.

dialysate (Δ cpm) increased with increasing reactivity of the recipient cells (Fig. 2). Ascher and Andron (6), and Hamblin and colleagues (7, 9) have subsequently published similar results in this system.

Comparison of Leukocyte Dialysates from Skin-Test Positive with those from Negative Donors. Mean augmentation ratios, as defined in the preceding section were 2.32 (range 0.4–6.5) with dialysate from skin-test-positive donors and 2.09 (range 0.5–5.6) with dialysate from skin-test-negative donors. Thus, dialysate from positive and negative donors produced similar augmentation ratios, which did not differ statistically.

In order to compare Δ cpm from positive and negative donors, it was necessary to correct for the differing reactivity of the various recipient cells to antigen alone. Regression lines were calculated separately for dialysates of positive and negative donors using the ln counts per minute obtained in the presence of dialysates and antigen (ordinate) vs. the ln counts per minute obtained with antigen alone (abscissa). The slopes and y intercepts for the lines of sensitive and nonsensitive donors were compared in an analysis of covariance.

A separate analysis was performed for each antigen, comparing dialysate preparations obtained from positive and from negative donors. Using this data, dialysates from sensitive and nonsensitive donors could not be distinguished from each other. Similarly, when the results obtained with positive dialysates for all three antigens were pooled and compared to all negative dialysates the two regression lines thus obtained did not differ significantly from each other in either slope or y intercept. Fig. 3 is a scattergram of the counts per minute with antigen in the presence or absence of dialysates from positive and negative donors.

To pursue this question further, we examined the sources of variation in the

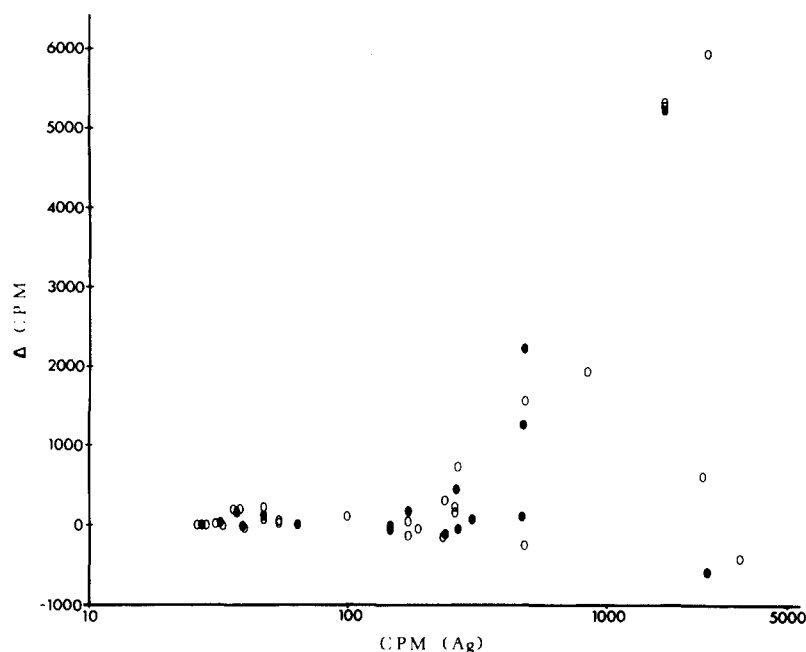


FIG. 2. $\Delta\text{cpm}(\text{cpm}[\text{dialysate} + \text{Ag}] - \text{cpm}[\text{Ag alone}])$ plotted against the cpm of [^{14}C]TdR incorporated into leukocytes cultured in the presence of antigen. Each point represents triplicate cultures. Closed circles represent dialysate from nonsensitive donors and open circles from sensitive donors.

culture system by assaying three different dialysates simultaneously on cells from three different recipients. An analysis of variance showed that the intrinsic level of lymphocyte reactivity was the major source of variation in the thymidine incorporation by such cells in the presence of dialysate. In fact, the component of variance due to the different dialysates was one-sixth that of experimental error while the component due to different recipient cells was four times greater than experimental error. The data from this experiment is summarized in Table II.

As shown in Table I, donor C. M.'s *in vitro* reactivities to the three antigens are markedly less than that of the other two donors. Donor C. M. was skin-test negative to 250 TU of tuberculin. Nevertheless, her dialysate augmented lymphocyte proliferation to tuberculin, as well as did the dialysates of the two more positive donors (B. S. and R. R.). This emphasizes again that the augmentation seen in these experiments is not a function of the dialysate donor's responsiveness to antigen.

Effect of Depletion of Antigen-Responsive Cells on Augmentation by Leukocyte Dialysate Preparations. Cell populations were specifically depleted of either PPD-, D.Tox.- or, SK-SD-responsive cells by triggering them with the respective antigen in the presence of lethal doses of [^3H]TdR. A representative experiment is shown in Tables II-V. Control cell populations were cultured with [^3H]TdR but no antigen as shown in Table II. Aliquots of lymphocyte populations treated in this fashion were then cultured subsequently with or without

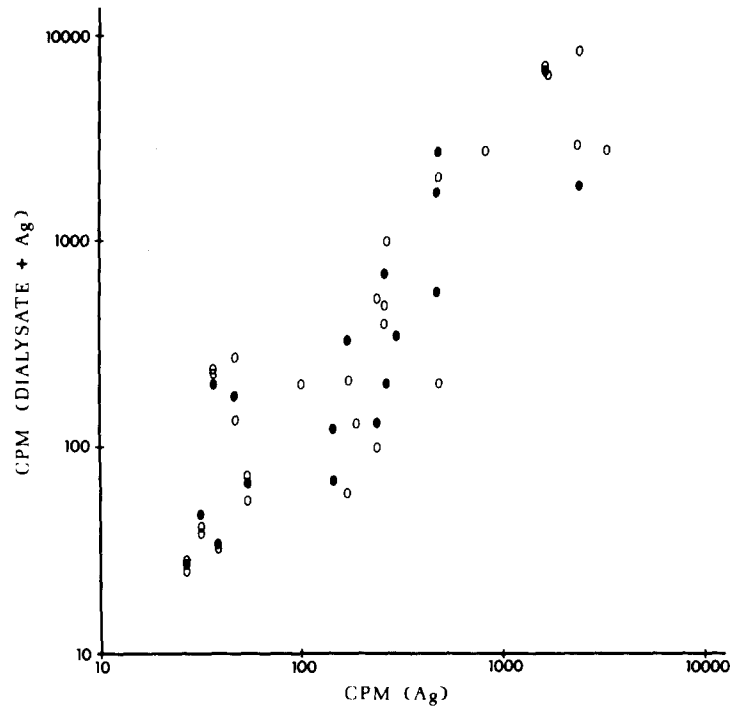


FIG. 3. [14 C]TdR incorporated by leukocytes cultured in the presence of antigen plus leukocyte dialysate plotted against the thymidine incorporated in the presence of antigen alone. Each point represents the geometric mean of triplicate cultures. Closed circles represent dialysate from nonsensitive donors and open circles from sensitive donors. The equations of the regression lines for dialysates obtained from sensitive and nonsensitive donors are $\ln y = 1.04 \ln x + 0.342$ and $\ln y = 1.03 \ln x + 0.271$, respectively. These lines are not significantly different from each other.

antigen and in the presence or absence of dialysate as shown in Tables III-V.

The stimulation ratio (cpm with Ag/cpm without Ag) in undepleted populations that were cultured without dialysate was used as a measure of "control" stimulation. This ratio was also calculated for undepleted populations cultured with dialysate and then expressed as a percentage of the control. Percentages of greater than 100 indicate augmentation over antigen alone by dialysate. Stimulation ratios were calculated for populations that were depleted of antigen-reactive cells and then cultured with antigen and with or without dialysate. These ratios were expressed as a percentage of the undepleted control. Percentages of less than 100 indicate reduced antigen-triggered proliferation after clonal deletion, regardless of the presence or absence of dialysate. Table VI summarizes the percentages obtained when lymphocytes were cultured both with and without dialysates, and includes experiments using eight dialysate preparations obtained from six donors and cultured with the cells of eight other individuals.

In control cultures, in the absence of dialysate, as seen on the left side of Table VI, the suicide technique significantly reduced the proliferative response to the specific antigen used with lethal thymidine but not to unrelated antigens. Thus,

TABLE II
CPM and Stimulation Ratios† Obtained when Three Different Leukocyte Dialysates were Simultaneously Assayed on Three Different Recipient Populations of Lymphocytes‡*

| Lymphocyte recipient | Dialysate donor | No anti-gen | PPD | D.Tox. | SK-SD |
|----------------------|-----------------|-------------|---------------|------------|------------|
| J. Ca. | None | 33 | 27 (0.8) | 32 (1.0) | 259 (7.8) |
| | R. R. | 29 | 28 (1.0) | 38 (1.3) | 485 (16.7) |
| | B. S. | 28 | 25 (0.9) | 41 (1.5) | 402 (14.4) |
| | C. M. | 27 | 27 (1.0) | 47 (1.7) | 693 (25.7) |
| A. S. | None | 23 | 1,678 (73.0) | 47 (2.0) | 37 (1.6) |
| | R. R. | 25 | 6,959 (278.4) | 135 (5.4) | 240 (9.6) |
| | B. S. | 26 | 6,990 (268.8) | 271 (10.4) | 232 (8.9) |
| | C. M. | 21 | 6,928 (329.9) | 175 (8.3) | 203 (9.7) |
| D. V. P. | None | 52 | 483 (9.3) | 54 (1.0) | 171 (3.3) |
| | R. R. | 35 | 166 (4.7) | 53 (1.5) | 59 (1.7) |
| | B. S. | 30 | 2,058 (68.6) | 73 (2.4) | 208 (6.9) |
| | C. M. | 45 | 2,693 (59.8) | 66 (1.5) | 332 (7.4) |

* Geometric means of triplicate cultures.

† cpm with antigen divided by cpm without antigen.

‡ The coefficient of variation for the data in this set of experiments (Tables II, III, IV, and V) is 8.3%.

lymphocytes from individuals sensitive to more than one antigen, responded normally to unrelated antigens whose clones were not deleted by this technique. In the presence of dialysate the proliferation of cells in response to the specific antigen was similarly impaired (right side of Table VI), whereas augmentation by dialysate of the responses to unrelated antigens was not affected.

For example, as is shown in Table VI, the population of cells that had been previously incubated with D.Tox. in the presence of lethal thymidine, when placed in secondary culture with D.Tox. plus dialysate responded at a level of 63% of control while the response of the same cells to SK-SD was 219% of control. Conversely, when the SK-SD-responsive cells were depleted, the response of these cells to subsequent culture with SK-SD and dialysate was 46% of control (SK-SD without dialysate), while the same cells, cultured with D.Tox. and dialysate had a stimulation ratio 37% of the control value (D.Tox. without dialysate). Thus, in reciprocal experiments, dialysate enhanced only the ongoing proliferation induced by antigen.

It should be noted that the boxed percentages in Table VI suggest that clonal deletion may have been incomplete. This impression is not correct and results from an artifact produced by averaging the data obtained from subjects who were nonreactive to the antigen (and hence had little or no change after the hot pulse) and those who had strong reactivity (whose responses were abrogated by thymidine suicide). When clonal deletions were not complete, dialysate produced a residual augmentation in the responsive cells that remained. Thus, *de novo* sensitization was not observed in this in vitro system.

Discussion

Our original observations using a macrosystem of lymphocyte culture to study the effects of leukocyte dialysates containing TF on blastogenesis provided

TABLE III
CPM and Stimulation Ratios† Obtained when Three Different Leukocyte Dialysates were Simultaneously Assayed on Three Different Recipient Populations of Lymphocytes from which the PPD-Responsive Cells had been Deleted‡

| Lymphocyte recipient | Dialysate donor | No anti-gen | PPD | D.Tox. | SK-SD |
|----------------------|-----------------|-------------|-------------|-----------|--------------|
| J. Ca. | None | 41 | 28 (0.7) | 29 (0.7) | 345 (8.4) |
| | R. R. | 30 | 24 (0.8) | 80 (2.7) | 517 (17.2) |
| | B. S. | 27 | 26 (1.0) | 88 (3.3) | 539 (20.0) |
| | C. M. | 28 | 24 (0.9) | 52 (1.9) | 688 (24.6) |
| A. S. | None | 564 | 807 (1.4) | 476 (0.8) | 242 (0.4) |
| | R. R. | 520 | 1,323 (2.5) | 393 (0.8) | 413 (0.8) |
| | B. S. | 633 | 1,372 (2.2) | 800 (1.3) | 342 (0.5) |
| | C. M. | 710 | 1,886 (2.7) | 644 (0.9) | 346 (0.5) |
| D. V. P. | None | 129 | 158 (1.2) | 182 (1.4) | 462 (3.6) |
| | R. R. | 209 | 1,748 (8.4) | 339 (1.6) | 626 (3.0) |
| | B. S. | 65 | 853 (13.1) | 164 (2.5) | 1,138 (17.5) |
| | C. M. | 105 | 652 (6.2) | 309 (2.9) | 798 (7.6) |

* Geometric means of triplicate cultures.

† cpm with antigen divided by cpm without antigen.

‡ The coefficient of variation for the data in this set of experiments (Table II, III, IV, and V) is 8.3%.

detailed evidence for the antigen dependence of the phenomenon and suggestive evidence of its concordance with the cutaneous reactivity of the donor (5). Palmer and Smith (16), Arala-Chaves et al. (17), and Basten et al. (18) have reported results that also suggest a role for the cutaneous reactivity of the donor in contributing to the results observed. In each of these reports it was emphasized that antigen specificity had not been established and that more detailed studies to evaluate the possible contribution of nonspecific adjuvant effects of crude leukocyte dialysates were required.

In the experiments reported here, the magnitude of the augmentation of antigen-stimulated lymphocyte proliferation, as measured by Δ cpm, occurring in the presence of dialysate was found to be a function of the underlying native reactivity of the recipient's lymphocytes to antigen rather than determined by the immune reactivity of the dialysate donor. When the differences in the baseline reactivity to antigen of the recipient's cells were adjusted by analysis of covariance, the augmentation effects of dialysates from sensitive and nonsensitive donors were identical. In contrast to Δ cpm, the augmentation ratio was found to be independent of the native reactivity of the recipient lymphocytes. Similar augmentation ratios were seen with dialysates from positive and negative donors. These results confirm and extend similar observations reported independently by Hamblin and co-workers (7, 9). In addition, we have observed that the dialysate by itself is not mitogenic and did not behave as an antigen in a lymphocyte culture system capable of detecting nanogram quantities of antigen (15).

The data provides evidence that, at least in this in vitro blood lymphocyte culture system, leukocyte dialysates augment the proliferative response to

TABLE IV
CPM and Stimulation Ratios† Obtained when Three Different Leukocyte Dialysates were Simultaneously Assayed on Three Different Recipient Populations of Lymphocytes from which the D.Tox.-Responsive Cells had been Deleted‡*

| Lymphocyte recipient | Dialysate donor | No anti-gen | PPD | D.Tox. | SK-SD |
|----------------------|-----------------|-------------|---------------|----------|--------------|
| J. Ca. | None | 85 | 56 (0.7) | 50 (0.6) | 432 (5.1) |
| | R. R. | 67 | 80 (1.2) | 59 (0.9) | 1,344 (20.1) |
| | B. S. | 167 | 103 (0.6) | 70 (0.4) | 1,196 (7.2) |
| | C. M. | 122 | 98 (0.8) | 77 (0.6) | 1,047 (8.6) |
| A. S. | None | 24 | 1,725 (71.9) | 24 (1.0) | 33 (1.4) |
| | R. R. | 30 | 3,623 (120.8) | 61 (2.0) | 40 (1.3) |
| | B. S. | 29 | 4,837 (166.8) | 31 (1.1) | 129 (4.4) |
| | C. M. | 26 | 4,708 (181.1) | 50 (1.9) | 127 (4.9) |
| D. V. P. | None | 24 | 381 (15.9) | 39 (1.6) | 73 (3.0) |
| | R. R. | 38 | 2,088 (54.9) | 30 (0.8) | 35 (0.9) |
| | B. S. | 40 | 3,408 (85.2) | 41 (1.0) | 443 (11.1) |
| | C. M. | 30 | 3,644 (121.5) | 42 (1.4) | 564 (18.8) |

* Geometric means of triplicate cultures.

† cpm with antigen divided by cpm without antigen.

‡ The coefficient of variation for the data in this set of experiments (Tables II, III, IV, and V) is 8.3%.

antigen only of precommitted lymphocyte populations, and do not initiate antigen reactivity in uncommitted cells. This interpretation is suggested by the observed increase in Δ cpm produced by addition of leukocyte dialysate plus antigen which parallels the level of the baseline reactivity of recipient lymphocytes to antigen alone. If, for example, a fixed number of uncommitted lymphocytes were engaged by materials in the dialysate, then the Δ cpm should remain constant and the ratio of counts per minute in the presence of dialysate plus antigen divided by counts per minute in the presence of antigen alone, should decrease as levels of baseline reactivity of recipient cells increased. In fact Δ cpm increased and the ratio remained unchanged. This finding is consistent with the interpretation that the dialysate exerts its effect only on lymphocytes that are already reacting to antigen, i.e., precommitted cells.

Direct evidence for this conclusion is provided by the demonstration that dialysates were unable to initiate or augment responses to a given antigen in populations of cells that had been previously depleted of lymphocytes capable of responding to that particular antigen. Taken together these data support the conclusion that the augmentation observed in this system arises from nonspecific adjuvant effects present in leukocyte dialysate prepared from nonimmune as well as immune individuals.

Leukocyte dialysates are known to contain a mixture of small molecules which may act to augment or to inhibit lymphocyte proliferation. A number of substances such as serotonin, ascorbic acid, and an unidentified cholinergic agent have been detected recently in such dialysates and shown to elevate cyclic GMP levels in human monocytes (19), and it is possible that the nonspecific effect of dialysate may be mediated via the monocyte. Increases in cyclic GMP

TABLE V
CPM and Stimulation Ratios† Obtained when Three Different Leukocyte Dialysates were Simultaneously Assayed on Three Different Recipient Populations of Lymphocytes from which the SK-SD-Responsive Cells had been Deleted‡*

| Lymphocyte recipient | Dialysate donor | No anti-gen | PPD | D.Tox. | SK-SD |
|----------------------|-----------------|-------------|---------------|------------|-----------|
| J. Ca. | None | 71 | 76 (1.1) | 149 (2.1) | 149 (2.1) |
| | R. R. | 144 | 70 (0.5) | 327 (2.3) | 330 (2.3) |
| | B. S. | 76 | 78 (1.0) | 418 (5.5) | 455 (6.0) |
| | C. M. | 58 | 60 (1.0) | 244 (4.2) | 329 (5.7) |
| A. S. | None | 24 | 2,117 (88.2) | 69 (2.9) | 24 (1.0) |
| | R. R. | 30 | 5,876 (195.9) | 154 (5.1) | 24 (0.8) |
| | B. S. | 28 | 5,159 (184.3) | 551 (19.7) | 38 (1.4) |
| | C. M. | 24 | 4,809 (200.4) | 407 (17.0) | 26 (1.1) |
| D. V. P. | None | 69 | 436 (6.3) | 64 (0.9) | 38 (0.6) |
| | R. R. | 37 | 3,418 (92.4) | — | — |
| | B. S. | 81 | 4,518 (55.8) | 84 (1.0) | 93 (1.1) |
| | C. M. | 36 | 3,457 (96.0) | 115 (3.2) | 110 (3.1) |

* Geometric means of triplicate cultures.

† cpm with antigen divided by cpm without antigen.

‡ The coefficient of variation for the data in this set of experiments (Tables II, III, IV, and V) is 8.3%.

TABLE VI
Failure of Leukocyte Dialysates to Restore the Response of Lymphocyte Populations Depleted of Antigen-Reactive Cells

| Antigen clone deleted | Secondary antigen without dialysates | | | Secondary antigen with dialysates | | |
|-----------------------|--------------------------------------|----------|---------|-----------------------------------|----------|----------|
| | PPD | D.Tox. | SK-SD | PPD | D.Tox. | SK-SD |
| None | 100* | 100 | 100 | 279 ± 58 | 221 ± 38 | 254 ± 50 |
| PPD | 33 ± 14 | 75 ± 16 | 82 ± 20 | 49 ± 13 | 152 ± 32 | 132 ± 39 |
| D.Tox. | 95 ± 17 | 58 ± 21 | 98 ± 25 | 316 ± 100 | 63 ± 10 | 219 ± 45 |
| SK-SD | 87 ± 15 | 106 ± 25 | 26 ± 6 | 294 ± 84 | 370 ± 90 | 46 ± 8 |

* Percent of control stimulation ratio; each number represents the mean of 6 to 16 experiments ± the SE. Boxed percentages are significantly less than 100%.

levels in lymphocytes, however, have been shown to result in proliferation which is antagonized by cyclic AMP (20-22). In addition, dialysates may contain a mixture of other small molecules (i.e. histamine, oligonucleotides, cold thymidine, calcium ions, etc.) which may act to augment or inhibit lymphocyte proliferation or [¹⁴C]TdR incorporation. The preponderance of one or another substance such as serotonin or histamine, in leukocyte dialysates will vary from preparation to preparation even from the same dialysate donor. This may account for the observation that a particular leukocyte dialysate may in some instances inhibit lymphocyte proliferation. The incorporation of [¹⁴C]TdR which occurs in the presence of dialysate probably reflects the summation of the effects of these agents, superimposed on the underlying reactivity of the recipients' lymphocytes to antigen.

Other attempts to develop an *in vitro* assay for TF activity using leukocyte dialysates have been reported in which lymphokine production is measured. These include the adaptation of the leukocyte capillary migration technique of S berg and Bendixen (23) which depends on the production of leukocyte inhibitory factor. Zabriskie and colleagues (24, 25) using leukocyte dialysates containing TF have reported on the conversion of nonimmune blood leukocyte populations to immune reactivity as measured by inhibition of leukocyte migration in the presence of tuberculin (PPD) or measles virus antigen. Similar results have been reported subsequently by Brandes and Goldenberg (26) using leukocyte dialysates from E-B virus-positive donors to confer inhibition of migration upon nonreactive cells in the presence of nasopharyngeal carcinoma antigens. However, the establishment of the immunological specificity of these *in vitro* effects of dialysates will also require more extensive study.

An *in vitro* assay for TF based on lymphocyte blastogenesis remains attractive because it would reflect the *in vivo* observation that after the administration of TF, the recipient's lymphocytes respond to antigen by proliferation as well as by lymphokine production. While the studies reported here highlight the capacity of the lymphocyte blastogenesis assay to selectively reveal the nonspecific, adjuvant activities of the materials present in crude leukocyte dialysates, it would be premature to conclude that the system cannot be refined to study the antigen-specific activities of TF. Basten et al. (18) have recently presented data to show that crude dialysates after purification by gel filtration yield a fraction which when assayed on recipient lymphocytes of weak intrinsic reactivity only causes augmentation of proliferation in the presence of the antigen to which the dialysate donor is reactive and not in the presence of antigen to which he is unreactive. If these preliminary findings are confirmed, the use of chromatographically purified fractions of dialysate may yet prove amenable to the study of the relationship between the antigen-specific and nonspecific activities of TF preparations both *in vitro* and *in vivo*. In any event the adjuvant effect of leukocyte dialysates on populations of lymphocytes proliferating *in vitro* should be considered in interpreting both the specific and nonspecific effects of TF reported in the course of clinical immunotherapy (2). The relation of these *in vitro* effects of leukocyte dialysates to such specific and nonspecific effects after administration of TF *in vivo*, remains to be elucidated.

Summary

After our initial report that leukocyte dialysates containing transfer factor augment the thymidine incorporation of antigen-stimulated lymphocytes, we have adapted the system to microleukocyte cultures. This modification permits both (a) the simultaneous assay of a single dialysate on the cells of multiple individuals, and (b) the assay of multiple dialysates on the cells of a single individual. The data thus secured, demonstrate that dialysates from both skin-test-positive and -negative donors produced similar degrees of augmentation whether the data are expressed as an arithmetic difference or as a ratio. When expressed as an arithmetic difference, the amount of augmentation is increased in proportion to the level of thymidine incorporation of the assay cells when they were stimulated by antigen alone. When expressed as a ratio, however, the degree of augmentation is independent of the response of the assay cells.

An analysis of the ability of dialysates to engage previously uncommitted lymphocytes and thus to augment thymidine incorporation, revealed that pre-committed cells were required. In these experiments, antigen-reactive cells were deleted from populations of peripheral blood lymphocytes by incubation with purified protein derivative of tuberculin, diphtheria toxoid, or streptokinase-streptodornase in the presence of [³H]thymidine of high specific activity. This deletion depressed or abolished the effect of dialysate on the residual population when it was recultured with the same antigen, but the effect on the response of the remaining lymphocytes to other antigens was unaltered.

In this study, leukocyte dialysate appeared to augment nonspecifically the thymidine incorporation of an antigen-specific precommitted clone of lymphocytes. The relationship of these adjuvant effects on peripheral blood lymphocytes *in vitro* to the specific and nonspecific activities of transfer factor *in vivo* remains to be elucidated.

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