Transfer of Cutaneous Hypersensitivity to Tuberculin with a Cell-Free Extract of Splenic Tissue.* (28917)

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It has been known for some time that delayed-type sensitivity to tuberculin(1) as well as late appearing reactions to simple chemical compounds(2) and to extracts or other micro-organisms besides mycobacteria (3,4) can be transferred passively to normal animals by means of viable cells derived from actively sensitized donors. The passive transfer phenomenon has been studied also as a possible mechanism in tumor immunity(5,6, 7) in the homograft reaction(8-11) and in autoimmune diseases(12,13). On the basis of a number of recent reports which suggest that the active principle in these transfer phenomena is subcellular(14,15,16,17,18,19), an effort was made to isolate an active transfer factor from lymphoid cells.

During these studies our attention was directed to the first supernatant fluid in which spleen cells were dissociated. In one series of experiments using serial increases in amounts of this supernate, when enough material was used it was found to possess transfer activity. This first supernatant fluid contains subcellular elements and is rich in proteins behaving like alpha and beta globulins. It is capable of passively transferring to a normal host a degree of tuberculin sensitivity comparable to that which can be accomplished with viable spleen cells from sensitized donors.

Material and methods. Sensitized animals. Guinea pigs, 300 to 600 g of several stock lines (Trapani-Campbell whites, Fitzsimons tan, Wright 13, mongrel) served as donors of cells and as normal recipients. Donors were immunized with Freund's adjuvant (Difco) containing Arlacel 15% and either 5 mg killed *Mycobacterium butyricum* (MB) or 10 mg of killed *Mycobacterium tuberculosis* (MT) per 10 ml of mineral oil. MB, 1 ml, was given in divided doses in the foot pads and subcutaneously in the nuchal region. Injections were repeated 14 and 20 days later using half the initial dose. MT 0.9 ml was given in a similar manner followed in 14 days by a single 0.1 ml nuchal injection.

Skin tests. Two to 4 weeks after the last sensitization injection, animals were tested intradermally with PPD (Parke Davis & Co.) or Old Tuberculin (OT) (Parke Davis 848). Erythema, induration and necrosis were recorded at 24 and at 48 hours in the donors. Skin test results in recipients were recorded at 30 minutes, and at 3, 6, 24 and 48 hours. OT reactions in donors exhibited diameters of induration of 10-30 mm accompanied by erythema. In some tests 3-5 mm of pale central edema appeared in 24 hours and progressed to necrosis in 48 hours.

Preparation of viable cell suspension and cell-free tissue extracts. Groups of 2-9 donors were exsanguinated by cardiac puncture, usually on the day the 48 hour skin test readings were made. Tissues were removed and processed aseptically at room temperature. Spleens (4-8 g) or lymph nodes (2-4 g) were placed in Tris-buffered Hanks' solution containing 200 mg% dextrose and 20-50 units/ml heparin (sodium Liquaemin, Organon). Fat and vessels were then dissected from the specimens following which they were transferred to a second Petri dish and the storage solution with its non-lymphoid tissue discarded. This Petri dish contained approximately 5 ml of the same solution per gram of tissue. Using small eye scissors, tissues were minced into the smallest possible pieces. These fragments were then further dispersed into a cloudy cell suspension with a glass syringe by multiple aspiration and discharges of the tissue suspension, care being taken to avoid bubbles and marked pressure changes. At this point the cloudy portion of the tissue suspension, without gross fragments, was transferred to a centrifuge tube. The fragments which could not be dispersed were dis-

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carded. The cell suspension was then centrifuged at 800 RPM (147 *g*) for 10 minutes to sediment the viable cells. These were set aside for transfer studies described below. The supernatant fluid hereafter designated as first supernate (I-S) was centrifuged in a fresh tube at 3500 RPM (2800 *g*) for 10 minutes. The sediment was discarded and the cleared fluid inspected microscopically to insure the absence of cells. The final I-S containing proteins, cell particles and other soluble elements was used in passive transfer studies (8-10 ml per recipient).

Lysed cells. Cells recovered from the I-S were washed 3 times by sedimentation at 800 RPM (147 *g*) from 3 changes of Hanks' solution. These cells were suspended in 5-10 ml Tris-buffered Hanks' solution and disrupted by 9 cycles of freezing (solid CO_2 and alcohol bath) and thawing (37°C). Cell debris was removed by sedimentation at 13000 *g* at 5°C for 30 minutes. The sediment was examined microscopically to insure disruption of all cells. The supernatant fluid and sediment from this treatment were used for passive transfer studies.

Transfer experiments. Normal guinea pigs, not previously skin tested, received experimental materials intraperitoneally. Tuberculin tests on untreated cage-mates were all negative.

Results. Data grouped in Table I give the experimental results. In early studies it was noted that repeated skin tests with 5 μg PPD (second strength) of either normal controls (experiments 5 a, b, c) or negative recipients (Exp. 9, 11, 13) did not induce tuberculin sensitivity. For this reason PPD was chosen as a test agent to follow the course of possible transferred sensitivity in the initial passive transfer studies. On the other hand, when sensitive donors were tested with both PPD and OT, OT 1:5 produced larger reactions than did 5 μg PPD. Accordingly, OT as well as PPD tests were tried in transfer studies (Exp. 11, 12, 19) which were then in progress using a variety of materials from sensitized donors. Even though the OT tests were done late after transfer, the results nevertheless were suggestive that a successful transfer with cell-free material had been accomplished (Exp. 12, 19 and 20). In the next experiments, therefore, OT was substituted for PPD as the initial test agent. In doing so, however, it was not possible to retest recipients with OT beyond the third day after transfer, as could be done with PPD, since OT will sensitize a normal animal in 7 days (Exp. 28, 31, 32) but not in 3 days (Exp. 91 a, b, c). Once it was found that OT could be used as a test agent, efforts were then directed toward increasing donor sensitivity. When heat killed *Mycobacterium tuberculosis* was substituted for killed *Mycobacterium butyrium* in the adjuvant, greater sensitivity in the donors was achieved, as is illustrated in example sets of the 2 types of donors give in Table I (MT Exp. 51 a, b, c; 58 a, b, c; MB Exp. 6 a, b). This greater sensitivity in cell donors then permitted the use of the more dilute 1:50 OT as a skin test agent in later transfer studies.

Attention was then directed toward the characteristics of the reaction which could be transferred with cell-free I-S material. Transfers done with I-S materials from 0.6 to 1.5 $\times$ 10^9 cells which contained 0.6 to 2.0 g% protein were accomplished when assayed by an initial OT skin test placed either 24 hours after transfer (Exp. 33, 34, 35, 80 a, b, c) on the third day after transfer (Exp. 48, 63 a, b, c) or on the seventh day after transfer (Exp. 43, 44). The reactions observed did not appear for 6 hours. They were characterized by erythema and induration of 5 mm mean diameter or greater, maximal between 24 and 48 hours. In appearance, size and time of occurrence, they were like reactions obtained in parallel experiments using viable cells from which the active I-S material was obtained. Both I-S and viable-cell transfer reactions were weaker than those in actively sensitized donor animals. Cell studies, unlike the I-S transfers, however, did not regularly give as good results in 24 hour skin tests (Exp. 77 a, b, c) as they did when the OT skin test was placed 3 days after transfer. The I-S transfers given in Table I are consecutive experiments of this type which have been regrouped for convenience in interpretation. In Exp. 39 the I-S material was passed through a 0.3 μ pore-sized millipore

TABLE I. Passive Transfer Studies.

Exp No.	No. cells ×10⁹	Materials transferred	Adjuvant	Induration mm mean diameter: 1st day	1st day 24′	1st day 48′	3rd day	3rd day 24′	3rd day 48′	7th day	7th day 24′	7th day 48′	14th day	14th day 24′	14th day 48′
Normal controls															
5a,b,c				PPD	—	—				PPD	—	—	PPD	—	—
7e,f,g,h,i				OT 1:5	—	—									
70a,b,c				OT 1:50	—	—									
91a,b,c				OT 1:50	—	—	OT 1:50	—	—						
Immunized donors															
6a			MB	PPD	12	5				PPD	12	9	OT 1:5	15	12
b			MB	PPD	9	3				PPD	8	5	OT 1:5	13	10
c			MB	PPD	7	0				PPD	9	7	OT 1:5	15	14
d			MB	OT 1:5	15	11									
e			MB	OT 1:5	9	10									
f			MB	OT 1:5	8	12									
51a			MT	OT 1:50	20	25									
b			MT	OT 1:50	22	17									
c			MT	OT 1:50	20	22									
58a			MT	OT 1:50	30	23									
b			MT	OT 1:50	20	18									
c			MT	OT 1:50	25	22									
Various subcellular elements															
8	0.24	I-S Imm Sp	MB	PPD	—	—				PPD	—	—			
9	0.22	I-S Imm LN	MB	PPD	—	—				PPD	—	—			
10	3.0	Dia S Imm Sp	MB	PPD	—	—				PPD	—	—			
11	3.0	DC Imm Sp	MB	PPD	—	—				PPD	—	—	OT 1:5	—	—
12	3.0	DC+Sed+I-S Imm Sp	MB	PPD	2	3				PPD	4	—	OT 1:5	8	7
13	3.0	Imm Sp Sed	MB	PPD	—	—				PPD	—	—			
14	1.5	DC Imm LN	MB	PPD	—	—									
15	1.5	DC+Sed+I-S Imm LN	MB	PPD	2	4									
16	1.5	Sed Imm Sp	MB	PPD	—	—							PPD	—	—
19	4.8	I-S Imm Sp	MB	PPD	2	—				PPD	4	7			
										OT 1:5	8	7			
20	2.8	I-S Imm Sp	MB	PPD	3	—				OT 1:5	10	8			
38	1.5	I-S Imm Sp	MB	PPD	4	3							PPD	3	—
													OT 1:5	8	3
39	1.5	I-S Imm Sp	MB	PPD	3	4							PPD	2	—
													OT 1:5	6	7

(Continued next page)

TABLE I (continued).

Exp No.	No. cells $\times 10^9$	Materials transferred	Adjuvant	Induration mm mean diameter: 1st day	1st day 24′	1st day 48′	3rd day	3rd day 24′	3rd day 48′	7th day	7th day 24′	7th day 48′	14th day	14th day 24′	14th day 48′
Viable spleen cells from immunized donors															
17	2.4		MB	PPD	4	—				PPD	5	4	OT 1:5	12	7
18	2.4		MB	PPD	2	—				PPD	5	2	OT 1:5	10	11
41	1.3		MB							OT 1:5	7	6			
42	1.3		MB							OT 1:5	5	5			
45	1.5		MT	OT 1:10	10	10									
60	1.0		MT	OT 1:5	8	7									
61	1.0		MT	OT 1:50	4	6									
62	1.0		MT	OT 1:50	5	—									
77a	1.2		MT	OT 1:50	—	—	OT 1:50	8	5						
b	1.2		MT	OT 1:50	—	5	OT 1:50	5	5						
c	1.2		MT	OT 1:50	—	9	OT 1:50	6	—						
First supernatant fluid from spleen cells from immunized donors															
22	1.5		MB	OT 1:5	9	8				OT 1:5	10	10			
23	1.1		MB	OT 1:5	10	8									
33	0.6		MB	OT 1:5	10	6									
34	0.6		MB	OT 1:5	8	5									
35	0.6		MB	OT 1:5	7	9									
80a	1.2		MT	OT 1:50	8	5									
b	1.2		MT	OT 1:50	6	—									
c	1.2		MT	OT 1:50	5	5									
48	1.5		MT				OT 1:10	8	7						
63a	1.0		MT				OT 1:50	7	6						
b	1.0		MT				OT 1:50	7	5						
c	1.0		MT				OT 1:50	5	—						
29	1.1		MB							OT 1:5	7	4	OT 1:5	6	7
43	1.3		MB							OT 1:5	5	—			
44	1.3		MB							OT 1:5	6	5			
First supernatant fluid normal spleen cells															
28	1.5									OT 1:5	—	—	OT 1:5	8	7
31	1.5			OT 1:5	—	—				OT 1:5	9	8			
32	1.5			OT 1:5	—	—							OT 1:5	—	—
40	1.1			PPD	—	—							PPD	—	—
73a	1.4			OT 1:10	—	—									
b	1.4			OT 1:10	—	—									
c	1.4			OT 1:10	—	—									

Legend: MB = *Mycobacterium butyricum* adjuvant. MT = *Mycobacterium tuberculosis* adjuvant. I-S = First supernatant fluid from spleen cell of immunized donor. I-S Imm LN = First supernatant fluid from lymph node cells from immunized donors. Dia S Imm Sp = Buffered saline fluid outside membrane after 48 hr of equilibration at 5°C with first supernatant fluid from immunized spleen cells. DC = Centrifuged supernatant fluid from cells disrupted by freezing and thawing. Sed = Sediment from disrupted cells. PPD = 5 μg.

membrane before transfer.

Transfer experiments were unsuccessful with spleen cell I-S from normal donors (Exp. 28, 31, 32, 40, 73 a, b, c). Animals skin tested with tuberculin received 0.1 ml of diluent in a control test site. None of these control tests were followed by measurable erythema or induration at 24 or 48 hours. Electrophoretic analysis of the I-S material indicates that it is rich in proteins which migrate in the α and β globulin positions. It also contains smaller amounts of other serum proteins among which are albumin and slow-moving globulins.

Discussion. The significant finding in this study is the successful passive transfer of reactions to old tuberculin using a cell-free extract of splenic tissue from tuberculin sensitive donors. The cutaneous reaction observed in these transfers did not appear until after 6 hours. The reaction was characterized by erythema and induration, maximal between 24 and 48 hours after the skin test was performed. In gross appearance, this reaction was similar to tuberculin reactions of comparable size induced either in animals passively sensitized by viable spleen cells or in the actively sensitized cell donors. Generally, donors reacted more strongly to OT than did passively sensitized recipients. Passively sensitized hosts did not exhibit necrosis in their reactions.

Evidently, there are multiple variables which may be of critical importance in achieving the results obtained with cell-free material in this series of experiments. Among these are an adequate degree of donor sensitivity, time of skin testing the donors, time of harvesting cells, number of cells from which the material transferred was derived and concentration of tuberculin used. On the other hand, similar problems have faced others who have studied passive transfer phenomena both with viable cells(20) and with subcellular materials. The present protocol appears to be another empirical procedure which can be used for study of the cellular transfer phenomenon.

It should be pointed out that the manner in which the splenic tissue was handled in these experiments for preparation of spleen extract, although gentle by mechanical standards, was quite traumatic from a biological viewpoint. Once tissue was reduced to fragments, the alternate aspiration and discharge of these through a syringe tip cannot help but disrupt many of the most fragile cells present. The materials released into the first supernatant fluid therefore were not only retained interstitial proteins to be found between and on spleen cells, but also a variety of subcellular elements. Furthermore, subsequent clearing of the spleen extract by centrifugation removed only the larger cell debris. Particles of microsomal size are presumed to be present in this material.

Although originally experiments were designed to increase the yield of proteins which behaved like α and β globulins in cell-free material, there is now no reason to believe that these proteins necessarily participate in the transfer phenomenon. The first supernatant evidently is a complex mixture of various subcellular particles, and soluble cellular compounds. Another protein or a nucleic acid which has survived the effect of tissue nucleases, or a protein-nucleic-acid-complex also might account for the transfer results. Fishman and Adler(21) who have passively transferred the induction of antibody formation with a subcellular extract of mononuclear cells, and Mannick and Egdahl(22) who have passively transferred the homograft reaction test with subcellular material have raised these possible mechanisms to explain their findings. In both of these studies, the passive transfer results were blocked by treating the transfer material with a ribonuclease suggesting that either a ribonucleic-acid or a ribonucleic-acid-complex might be the active principle. Explorations now in progress of the possible role of a nucleic acid, protein or nucleic-acid-protein complex in our transfer studies do not yet permit speculation regarding the identity of the transfer mechanism.

Summary. 1. A method is described for preparing a cell-free first supernatant fluid from dissociated spleen cells from guinea pigs sensitized to tuberculin with Freund's complete adjuvant. 2. Intraperitoneal injections of this cell-free material confer on normal guinea pigs an ability to react in a delayed

fashion to the intracutaneous injection of old tuberculin. This reaction is similar to that which can be transferred passively by the viable cells from which the cell-free supernatant fluid is derived.

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Iodide Concentrating Ability of Normal Human Thyroid Cells in Tissue Culture.* (28918)

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Studies on the iodide-concentrating ability of isolated thyroid cells have recently been published. Pulvertaft *et al*(1) reported that cells obtained from pathologic human thyroid cells were able to concentrate iodide, although no values for cell-to-medium iodide concentration ratios were given. Pastan(2) found that isolated calf thyroid cells were able to incorporate I-131 into iodinated proteins, although no data concerning iodide-concentration ability were reported. Tong and coworkers(3) reported cell-to-medium iodide concentration ratios of 6-9, using isolated sheep thyroid cells. This study was initiated to investigate the iodide-concentrating ability of normal human thyroid cells in tissue culture.

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Materials and methods. Normal thyroid tissue was obtained from radical neck dissections for cancer of the head and neck. Other normal thyroid tissue was obtained from lobectomies performed because of solitary nodules of the thyroid gland. All tissue was examined histologically for verification of the presence of normal thyroid tissue.

The method for tissue culture of isolated human thyroid cells was that of Irvine(4), with modification as previously published(5). Thyroid tissue was obtained at surgery and immediately transported in cold sterile Delbeccos saline solution containing potassium penicillin G (100 units/ml), and streptomycin sulfate (100 μg/ml). Cells were obtained by trypsinization at pH 8.0 after trimming, mincing and washing of the thyroid tissue. The trypsinizing fluid consisting of 0.25%