CELLULAR TRANSFER OF TUBERCULIN TYPES OF HYPERSENSITIVITY; PROSPECTS FOR RECONCILIATION WITH SEROLOGIC RESULTS[1]

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Anergy to lepromin, and consequent Mitsuda negativity, has long been held to be of significant import in the prognosis and classification of human leprosy. It is widely held that this anergic state reflects absence of the ability to respond to or resist infection with *Mycobacterium leprae*. There are, however, reasons for questioning the absolute validity of this concept.

Despite the prodigious numbers of bacilli present in the lesions of well-established lepromatous leprosy, the progress of this form of the disease is typically restrained, and some patients may accomplish spontaneous recovery. Because of the marked disparity between macroscopic lepromin positivity and histologic demonstration of the tuberculoid granuloma, and because some recovered lepromatous individuals recover reactivity to Mitsuda testing, Azulay *et al.* were forced to conclude that lepromatous leprosy does not necessarily arise in the anergic margin of the population which lacks the capacity of responding to lepromin with tuberculoid granuloma formation (1).

In the more fulminating disease produced in murine animals by *M. leprae murium*, there is evidence of continuous enhancement of resistance. In this disease, as in human leprosy, early rates of increase in leproma weights are not maintained. Further, infection of rats and mice *in utero* or neonatally markedly raises their resistance to subsequent challenge (23). Hanks (8) has demonstrated healing of rat lepromas late in the course of disease, again indicating that resistance increases during the progress of infection. There is an abundance of other direct evidence that resistance develops during the course of mycobacterial infection (10, 18). Finally, white cells from peritoneal exudates of rats infected with *M. leprae murium* transfer the delayed type mycobacterial hypersensitivity to normal guinea-pigs (22). This capacity increases after second challenge. These observations indicate that even though the microorganisms cannot be readily overcome (8), general cellular response to specific antigens is enhanced during this type of infection. They also indicate that different levels of cellular response can be assayed in animals which do not exhibit classical dermal sensitivity.

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The transfer of sensitivity from animals relatively anergic on dermal skin testing to an indicator species, immediately suggested the possibility of using this technique for examining experimentally the concept that patients with lepromatous leprosy have lost their ability to respond to *M. leprae* antigens. The possibility exists that the failure of elicitable dermal response in lepromatous leprosy patients may be due, at least in part, to the necessity of injecting antigen into cutaneous tissues of individuals who already possess large reservoirs of antigen or whose skin is anergic for some other reason. It therefore seemed desirable to measure, by means of passive transfer techniques, the reactivity of cells derived from other than cutaneous tissues.

Before applying the local passive transfer technique to the lepromatous leprosy problem, however, it was deemed advisable first to ascertain the ability of leucocytes from tuberculin-positive individuals to incite tuberculin-type hypersensitivity when mixed with tuberculin and injected into the skin of normal animals. Thus, Bacon *et al.* ([2]) have shown by the transfer method that cells of rats sensitized to tubercle bacilli are hyperreactive—a fact shown by at least three other workers or groups in the last several years ([5, 12, 21]). The present report will deal mainly with results obtained in interspecies transfer experiments using human circulating leucocytes.

MATERIALS AND METHODS

Cell donors.—A series of 35 medical students, or patients on the Medical Outpatient Service of Hubbard Hospital, who gave positive reactions to PPD were used as donors of sensitive cells. A group of 11 individuals from the same source who were negative to second strength PPD (250 STU) were employed as donors of normal cells.

Preparation of leucocytes.—White cells were isolated from 10 cc. of heparinized blood by sedimenting erythrocytes according to the fibrinogen method of Skog and Beck[19]. Cells were washed twice in heparinized Hanks' balanced salt solution (BSS); packed volumes were noted (usually 0.1 cc.); leucocyte counts were done, viability was determined, and smears for differential counts were prepared. Suspensions were then divided into 2 equal volumes, centrifuged, and the supernatant fluids were discarded.

Test Procedures.—Testing was carried out essentially as reported previously ([22]). Cell aliquots were resuspended in either 0.15 cc. of BSS or 0.15 cc. of Massachusetts State or Parke-Davis Old Tuberculin diluted 1:15 in BBS. Rabbits, obtained from a local supplier, were injected intradermally with Cell-OT and Cell-BBS mixtures and with OT alone. Leucocytes were injected within 4 hours after their removal from donors, and the usual test site carried 0.05 cc. of packed cells 80 per cent of which were viable. Differential counts revealed the average population to consist of about 65 per cent mononuclear cells.

Areas of skin reaction were determined at 24 and 48 hours. Specific reactivity was judged by subtracting the area of reaction produced by a cell-BSS mixture from that produced by the corresponding cell-OT mixture.

Serologic procedures will be described where indicated.

RESULTS

Fig. 1 illustrates a typical test scheme, with the results obtained. It can be seen that OT 1:5 stimulated no recipient response, but that

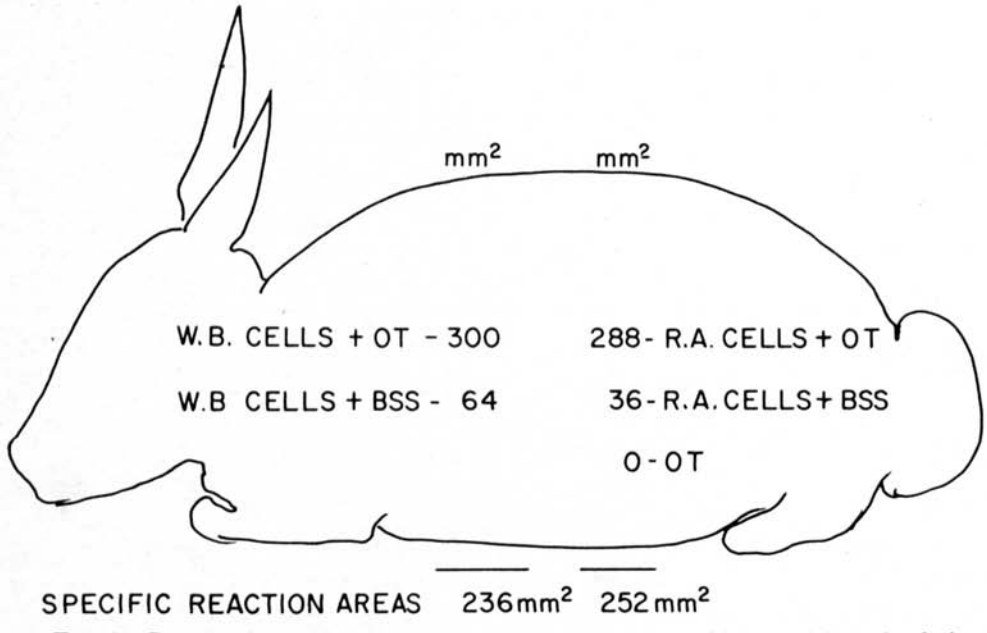

FIG. 1.—Representing 48-hour cutaneous responses in the rabbit receiving circulating leucocytes from donor "W.B." or "R.A." (tuberculin-sensitive human subjects), mixed with Old Tuberculin (OT) or balanced salt solution (BSS).

when OT was mixed with cells from a sensitized person a significantly greater response was elicited than by cells plus BSS.

Control sites were characterized by raised areas with dark centers and peripheral blanching due to the volume of injected cells. Also, slight erythema surrounded the raised areas. More pronounced erythematous zones, accompanied by slight thickening and induration, characterized cell-OT sites.

Table 1 presents representative results of the transfer experiments, and illustrates the range of specific reactivity obtained. Cells from tuberculin-negative individuals, when mixed with OT, did not elicit reaction sites that were greater than the corresponding areas of cells plus BSS.

The bar graph in Fig. 2 shows the average reactions obtained in response to the three types of injection mixtures decribed. Cells from sensitive individuals uniformly gave evidence of heightened reactivity when mixed with tuberculin.

While no serologic studies have been made with sera from the individuals involved in the transfer studies described, certain findings in our laboratory using sera from other subjects infected or immunized with mycobacteria are pertinent to the present discussion.

Of 20 sera obtained from rats infected with *M. leprae murium* (*Mlm*), with marked demonstrated ability to resist a second infection of that organism and with cellular hypersensitivity, only 4 gave evidence

TABLE 1.—*Range of 48-hour reactions obtained in dermal response of normal rabbits to leucocytes from tuberculin-positive and tuberculin-negative human subjects, plus Old Tuberculin or balanced salt solution, and to OT alone.*

Cell donor	48-hour reactions (mm^2)			
	Cells + OT	Cells + BSS	OT alone	Specific area
Tuberculin-positive individuals				
F.E.	300	100	0	200
L.H.	400	49	0	351
J.B.	176	36	0	140
2.B.	140	25	0	115
D.R.	288	16	0	272
R.J.	420	64	0	356
B.A.	300	25	0	275
Tuberculin-negative individuals				
C.E.	64	64	0	0
G.W.	56	56	0	0
G.J.	81	81	0	0

of mycobacterial antibody. One serum reacted with polysaccharide from OT, and another with protein antigens from OT, in hemagglutination tests. The gamma-2 globulin fractions of 2 different sera were positive following fractionation by the method of Nichol and Deutsch ([11]), when tested in tanned-cell hemagglutination tests against antigens contained in *Mlm* sonicates.[3] Selected sera from this group were also negative in Parlett's agar diffusion system ([13]) against *Mlm* sonicates. However, hyperimmune rabbit serum prepared against the antigens present in *Mlm* sonicates reacted strongly in diffusion systems, giving 4 distinct bands.

The sera of 60 rats injected with killed H37Rv and possessing sensitive cells, were all negative when tested with polysaccharide or protein antigens from OT by hemagglutination procedures. All of the sera tested, however, showed low levels of complement-fixing antibodies against OT. None of them had demonstrable complement-fixing antibody for any of a variety of normal mouse-tissue antigens.

A summation of results obtained in serologic studies with 10 lepromatous and 2 tuberculoid leprosy sera is presented in Table 2. Positive hemagglutination was demonstrated against polysaccharide antigens from OT and against proteins from both OT and *Mlm* sonicate. However, no correlation could be made between the titer to a given antigen and the clinical state of the patient.

[3]Mlm-infected tissues were homogenized and bacilli were washed free of tissue components. A 10 per cent suspension of washed bacilli was subjected to sonic oscillation in a 10 K.C. Raytheon Sonic Oscillator until no intact bacilli were demonstrable. Supernatant fluids from such preparations have been termed "sonicates" and were used in hemagglutination and agar diffusion experiments.

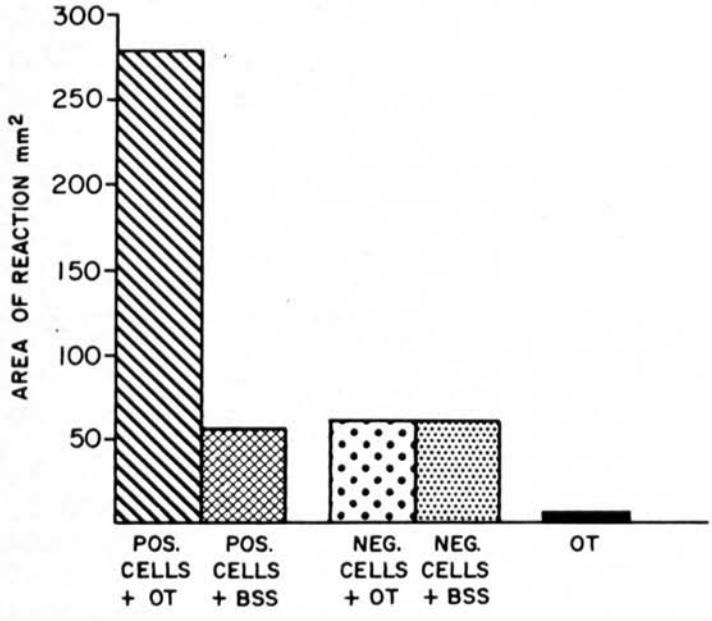

FIG. 2.—Average 48-hour skin reactions in normal rabbits to the intradermal injection of mixtures of Old Tuberculin (OT) or balanced salt solution (BSS) and cells from tuberculin-positive and tuberculin-negative human subjects.

Employment of a 1:20 dilution of Lederle quadruple-strength OT in Parlett's agar diffusion system allowed the detection of reaction bands with 5 of the 7 lepromatous sera tested. It is of interest that only the sera from lepromatous cases were positive in this limited series.

Five sera tested by the indirect fluorescent antibody technique gave relatively weak, but definitely positive staining of both *Mlm* and H37Rv.

TABLE 2.—*Serologic reactivity of leprosy sera.*

Patient[a]	Hemagglutinin[b]			Agar diffusion[c]	
	OT polysaccharide	PPD protein	*Mlm* protein	OT	*Mlm*
1. Lep +	256	64	16	+	−
2. Lep +	64	128	8	+	+
3. Lep +	16	64	128	ND	ND
4. Lep +	64	128	64	+	+
5. Lep +	8	128	64	ND	ND
6. Lep +	32	8	4	ND	ND
7. Lep +	16	16	64	+	+
8. Lep −	64	64	128	+	−
9. Lep −	256	256	128	−	−
10. Lep −	4	32	32	−	−
11. Tub −	64	128	128	−	−
12. Tub −	32	64	32	−	−

[a]Lep = lepromatous; Tub = tuberculoid; (+), (−) = presence or absence of bacilli in smears of skin scrapes.

[b]Titers expressed as reciprocal of dilution; all sera previously absorbed with normal red cells.

[c](+), (−) = presence or absence of 1 to 2 distinct bands after 14 days' incubation; ND = not done.

Limited experience with passive cutaneous anaphylaxis using leprosy sera and antigens from tuberculin has, as yet, not yielded positive results.

Also, leprosy sera have shown low levels of complement-fixing antibody for a variety of normal human tissues, including kidney, testes, liver, spleen, lung, and heart.

DISCUSSION

The independence of tuberculin sensitivity and the presence of hemagglutinating antibody against tuberculin is a well-established fact ([7]) which has been reemphasized by certain facets of the studies reported here. Indeed, in general the state of delayed hypersensitivity develops and persists independently of humoral antibody-production ([3, 6]). There is little reason to doubt that this truism is operative in leprosy.

Obviously, this does not mean that "cellularists" are "cellularists" and "humoralists" are "humoralists," and "never the twain shall meet."

Hanks ([8]) concludes that high levels of cellular response are required effectively to combat mycobacterial disease. Whether this response be one of cellular sensitization or of antibody synthesis, knowledge concerning the role of the one contributes to a better understanding of the part played by the other.

Results of Taylor *et al.* ([20]) indicate that late response to lepromin does not adequately test the capacity for immune response. Also, the use of heat for the preparation of lepromin has been questioned ([9]).[4]. In addition, we would raise the question as to the complete validity of evaluating lepromatous patients by the injection of antigen into anergic sites. The local passive transfer technique could overcome this latter objection by the use of systemic cells and soluble antigens from *M. leprae.* The use of this procedure with cells from *Mlm*-infected rats showed that tuberculin resulted in better reactivity than did *Mlm* lepromin, presumably because of the more ready availability of cross-reacting antigens in the soluble mycobacterial material ([22]). Taylor *et al.* ([20]) also emphasize the need for a specific leprolin preparation and recommend the substitution of a specific Fernandez-type reaction for the Mitsuda test in the evaluation of ability to respond.

Agar diffusion, as developed by Parlett and Youmans ([14, 15, 16]), Parlett *et al.* ([17]), and Parlett ([13]) for tuberculosis systems, would appear to offer an extremely useful tool for the identification and eventual isolation of specific antigens from *M. leprae.* Such materials, if protein, could well provide specific antigens for use in direct and passive trans-

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[4]Disruption of washed bacilli by sonic oscillation or by treatment in a Ribi cell fractionator could provide antigens from *M. leprae* with minimal modifications in the native antigenic moiety.

fer skin tests. These workers observed no cross reactivity in agar between 13 leprosy sera and unheated antigens from cultures of tubercle bacilli. Similarly, Weiss and Fusillo ([24]) found no reactivity between tuberculosis antigen and leprosy sera by the globulin titration technique. On the other hand, Burrell and Rheins ([4]), using OT as antigen in agar plate diffusion studies, demonstrated reactivity with a pool of lepromatous sera and obtained negative results with tuberculoid sera. These results closely parallel our own, and possibly reflect loss of specificity in the heated antigen employed.

Also, the demonstration of tissue-reacting antibody in certain leprosy sera suggest the interesting possibility of the operation of an autoimmune mechanism in this disease.

SUMMARY

1. The validity of Mitsuda testing for the evaluation of the ability of leprosy patients (especially lepromatous cases) to respond is questioned. A passive transfer technique has been described for the possible circumvention of certain of the objections raised.

2. Results of hemagglutination, complement fixation, agar diffusion, and fluorescent antibody tests using human and animal sera *versus* mycobacterial antigens suggest that with the possible exception of agar diffusion techniques, results of serologic tests do not correlate with the clinical status of individuals infected with mycobacteria.

3. The possible usefulness of serologic studies for providing specific antigens of value in determinations of cellular sensitivity has been considered.

RESUMEN

1. Pónese en duda la validez de la comprobación de Mitsuda para justipreciar la capacidad de los leprosos (sobre todo casos lepromatosos) para reaccionar. Se describe una técnica de transferencia pasiva para el posible desenredo de los reparos ofrecidos.

2. Los resultados de la hemaglutinación, la fijación del complemento, la difusión del agar y los ensayos de anticuerpos fluorescentes, usando sueros humanos y animales, compardos con los de los antígeno micobacterianos, sugieren que, con la posible excepción de las técnicas de difusión del agar, los resultados de las serorreacciones no correlacionan con el estado clínico de los individuos infectados con micobacterias.

3. Se considera la posible utilidad de los estudios serológicos para el suministro de antígenos específicos de valor en las determinaciones de la sensibilidad celular.

RESUMÉ

1. Le bien-fondé de l'épreuve de Mitsuda aux fins d'évaluer l'aptitude des lépreux (et particulièrement des lépromateux) à réagir, est mise en question. Une technique de transfert passif a été décrite, qui pourrait éventuellement tourner certaines des objections avancées.

2. Les résultats de l'hémagglutination, de la fixation du complément, de la diffusion sur agar, et des tests aux anticorps fluorescents, faisant usage des réactions de sérums humains et animaux à l'égard d'antigènes mycobactériens, suggèrent que les résultats des tests sérologiques, à l'exception peut-être de la diffusion sur agar, ne correspondent pas à l'état clinique des individus infectés par des mycobactéries.

3. On discute de l'utilité possible des études sérologiques pour mettre au point des antigènes spécifiques valables pour déterminar la sensibilité cellulaire.

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