SOME IMMUNOLOGIC PROPERTIES OF CELLS OF GUINEA-PIGS INOCULATED WITH *MYCOBACTERIUM LEPRAEMURIUM* [1, 2]

WALDEMAR F. KIRCHHEIMER, M.D.
and
WILLIAM J. COOK, JR., M.S.
United States Public Health Service Hospital
Carville, Louisiana

INTRODUCTION

At the VIIIth International Congress of Leprology in Rio de Janeiro in September 1963, the members of the Panel on Lepra Reaction expressed the opinion that this type of reaction is mediated by some as yet obscure form of hypersensitivity. They recommended that intensive immunologic research be undertaken to clarify the pathogenesis of the reaction ([26]). It is generally believed that the clinical and histologic manifestations of leprosy reflect various degrees of host resistance against infection with *Mycobacterium leprae* ([27]). In reality, however, nothing is known about the nature of this resistance. Failure of leprosy bacilli to multiply, following inoculation, may be the result of either native or acquired characteristics of host tissue or of a combination of both. As in other mycobacterial infections, there is no evidence that resistance to Hansen's bacillus depends on participation of ordinary circulating antibodies ([4, 17]). The existence of an infectious type of hypersensitivity in tuberculosis and its relation to the pathogenesis of the disease have been firmly established and well described ([28]). The Fernández reaction ([9]) seems to be the only evidence that can be adduced in support of the view that this type of hypersensitivity is involved in leprosy. One of the characteristics of the infectious type of hypersensitivity is the frequently corroborated observation that antigens identical with or similar to the engendering proteins exert toxic effects *in vitro* on cells of hypersensitive, but not of normal individuals ([1, 8, 10, 22, 24, 29, 33]). In addition, it is well known that this type of hypersensitivity can be transferred from hypersensitive individuals to normal individuals of the same or another species with cells subject to the toxic effects of the antigen ([3, 6, 12, 14, 35]).

There is some evidence that mononuclear leucocytes of tuberculin-sensitive guinea-pigs are metabolically more active than cells of the same kind obtained from normal animals ([25]). *In vitro* addition of antigen does not seem to alter the respiration of leucocytes of sensitive animals ([16, 20]). With the exception of successful heterologous passive transfer of hypersensitivity to antigens of *Mycobacterium leprae-*

[1]Received for publication June 9, 1964.
[2]This research was financially supported by the Division of Hospitals, United States Public Health Service.

murium by Wallace ([34]), there is no information on immunologic and metabolic characteristics of mononuclear cells of animals infected with this microorganism.

The experiments reported in this publication deal with homologous passive transfer of sensitivity to mycobacterial antigens with living cells of guinea-pigs inoculated with the bacillus of rat leprosy, and the toxic effect of a mycobacterial antigen for migrating cells of explants from their spleens. A later publication will deal with the effects of such antigens on the respiration of their mononuclear leucocytes.

MATERIALS AND METHODS

Mycobacterial antigens.—Mycobacterium phlei, Mycobacterium species 607 and *Mycobacterium bovis* (BCG strain) were grown on the surface of Proskauer-Beck medium for 4 weeks in the case of the mammalian organism and for one week in the case of the saprophytes. The surface growth was removed to a mortar by means of a large wire loop and ground in normal saline. *M. lepraemurium* suspension was prepared from subcutaneous lepromas of C_{57} black mice infected three months previously with the Hawaiian strain of the microorganism. The lepromas were minced with a pair of scissors and ground in a mortar with sterile sea sand with addition of Hanks' balanced salt solution (BSS). The resulting tissue-bacterial suspension was centrifuged at 50°C and 1,000 to 2,000 r.p.m. until acid-fast stains of the supernatant contained mainly acid-fast bacteria and a minimum of tissue fragments.

A suspension of *M. leprae* was prepared in the same manner, except that the source of this material was liver from a case of lepromatous leprosy.

The bacterial cells were treated in a Raytheon sonic oscillator, model D F, at 10 K C until only occasional acid-fast particles could be found.

After this treatment the preparations were centrifuged at 4,000 × G at 5°C for 45 minutes. The supernatants were removed and dialyzed against distilled water at 5°C for 72 hours. The antigen-containing solutions were sterilized by Seitz filtration. Nitrogen determinations were carried out by microkjeldahl analysis, using steam distillation. The following amounts of nitrogen were found in 0.1 ml. of the respective antigens, the amount used for skin testing:

Antigen	*Mgm. N/0.1 ml.*
Phlei	0.022
607	0.036
Leprae	0.033
BCG	0.010
Lepraemurium	0.003

Inoculation of guinea-pigs.—Mature albino guinea-pigs of either sex were used as donors of normal and sensitive cells. Animals serving the latter purpose received two subcutaneous inoculations of approximately one billion *M. lepraemurium* one week apart from 3 to 4 months prior to cell harvest. The suspensions of *M. lepraemurium* were prepared from mouse lepromas as described above. Bacterial counts were made by a method similar to that described by Shepard ([31]). Approximately one week before use these guinea-pigs were skin-tested with the mycobacterial antigens and with 0.005 mgm. of PPD. The results of skin tests on inoculated animals are summarized in Table 1. Normal guinea-pigs did not respond with typical reactions to any of these antigens.

Mononuclear cells from peritoneal exudates.—Normal guinea-pigs and guinea-pigs inoculated with *M. lepraemurium,* which served as donors of mononuclear cells in passive transfer studies, were given 15 ml. of a light paraffin oil intraperitoneally 48 hours before cell harvest. Their peritoneal cavities were washed out with BSS containing 0.03 per cent of heparin and 0.5 per cent of bovine albumin. The washings were added to separatory funnels to separate them from the paraffin oil. The pooled washings were

added to siliconized 250 ml. centrifuge tubes and centrifuged for 10 minutes at 1,000 r.p.m. The supernatants were drawn off and discarded. Following resuspension in BSS and recentrifugation, the cells were transferred with siliconized capillary pipettes to siliconized screw-capped tubes and resuspended in BSS. Recipient guinea-pigs received intraperitoneally 2 ml. of this suspension, containing approximately 250-400 million cells. The cellular composition of these suspensions, determined from Wright-stained preparations, was as follows: monocytes, 50-80 per cent, lymphocytes, 20-50 per cent, granulocytes, 1-3 per cent. Viability was determined by supravital staining with neutral red and Janus green ([23]). Most cells appeared viable.

Splenic cells.—Spleens of sensitized and normal guinea-pigs were minced in BSS. The homogenate was centrifuged for 10 minutes at 1,000 r.p.m. The upper grayish-appearing cell layer was removed to 15 ml. centrifuge tubes. Following centrifugation for 10 minutes at 1,000 r.p.m. the supernatants were discarded and the cells resuspended in BSS. Recipient guinea-pigs received an intraperitoneal injection of 2-3 ml. of this suspension, containing approximately 0.5 ml. of packed cells.

Cytotoxicity experiments.—Washed spleens of sensitized and normal guinea-pigs were cut into pieces 1 to 2 mm. wide. After repeated washing in cold BSS they were transferred with capillary pipettes to the flat surface of Leighton tubes and 1.0 ml. of fresh normal guinea-pig plasma was added. If exposure to antigen was desired, the added plasma contained phlei antigen at a concentration of 0.022 mgm. N per ml. Explants of the spleens of normal and of sensitive guinea-pigs in the presence and absence of added antigen were incubated for 24 hours at 37°C. Micrometer measurements of the width of the zone of cell migration were then made on the four edges of each explant. For each explant the average distance of migration was determined from these measurements and expressed in micrometer units. One micrometer unit equals 20 microns.

RESULTS

The data summarized in Table 1 show that after inoculation with *M. lepraemurium,* guinea-pigs develop delayed-type cutaneous reactivity to soluble antigens prepared from *M. lepraemurium, M. species* 607, *M. phlei,* and *M. bovis* (BCG), as well as toward PPD. The *M. leprae* antigen failed to elicit skin reactions. It does not seem warranted to conclude that there is no cross sensitization between *M. leprae* and *M. lepraemurium.* The high nitrogen content of the lepra antigen may be misleading, since it is not known how much of this was contributed by the bacteria. It seems not unreasonable to suspect that most of the nitrogen in this preparation was derived from human liver. The skin reactions with phlei antigen were strongest and least variable. Therefore this antigen was used in the cytotoxicity studies.

TABLE 1.—*Skin reactions of guinea-pigs inoculated with* M. lepraemurium *to soluble mycobacterial antigens.*

Antigen	No. of animals tested	No. positive reactions	Means of average diameters of reaction in mm. at 24 hrs.
M. lepraemurium	16	14	11 ± 4
M. leprae	16	0	—
M. species 607	9	4	6
M. phlei	16	16	27 ± 3
M. bovis (BCG)	16	16	24 ± 6
PPD	4	4	14 ± 3

TABLE 2.—*Number of skin-reactors to mycobacterial antigens 48 hours after homologous transfer of living cells from guinea-pigs inoculated with* M. lepraemurium.

Material transferred	Antigens			
	M. leprae	*M. lepraemurium*	*M. bovis* (BCG)	*M. phlei*
Peritoneal cells of inoculated guinea-pigs	0[a] / 3	0 / 8	7 (12 ± 3)[b] / 9	7 (16 ± 2) / 7
Peritoneal cells of normal guinea-pigs	0 / 4	0 / 4	0 / 4	0 / 4
Splenic cells of inoculated guinea-pigs	0 / 7	0 / 9	4 (9 ± 4) / 7	7 (13 ± 4) / 7
Splenic cells of normal guinea-pigs	0 / 4	0 / 4	0 / 4	0 / 4

[a] number of reactors / number of recipients tested

[b] Means of average diameters of positive reactions in mm. at 24 hours.

The results of the passive transfer experiments are summarized in Table 2. Recipients of peritoneal macrophages and splenic cells of inoculated guinea-pigs reacted regularly to skin test with phlei antigen 48 hours after cell transfer. Their skin reactions at 24 hours were larger than those elicited by BCG antigen. In addition, this antigen did not evoke positive reactions in all recipients of sensitizing material, particularly the ones that had received splenic cells. Neither leprae nor lepraemurium antigen caused skin reactions in guinea-pigs that had received cells of infected donor animals.

In view of the data presented in Table 1 it might be concluded that the lepraemurium antigen was too weak to elicit reactions in passively sensitized guinea-pigs, since it caused only comparatively small reactions even in the actively sensitized cell donors. On the basis of the same reasoning the negative results with leprae antigen are not surprising.

Recipients of cells from normal guinea-pigs did not react to cutaneous injections of any of the antigens. These results clearly show that the sensitivity of guinea-pigs against mycobacterial antigens following in the wake of inoculation with *M. lepraemurium,* can be transferred to normal guinea-pigs with living mononuclear cells of induced peritoneal exudates and with their splenic cells.

The results of the cytotoxicity experiments are summarized in Table 3. This table lists, for explants of 6 normal and 6 sensitive animals, in the absence and presence of antigen, the cell migration in micrometer units and the number (n) of explants of each spleen from which the distance of migration was calculated. For each of the six experiments listed a migration index was calculated by dividing the distance of migration in the presence of antigen by the distance of migration in the absence of antigen. This was done so that account

TABLE 3.—*Effect of phlei antigen on cell migration from splenic explants of normal guinea-pigs and guinea-pigs inoculated with* M. lepraemurium.

Migration[a] with antigen	n[b]	Migration[a] without antigen	n[b]	Migration index
		Explants from normal guinea-pigs		
39.0	6	34	6	1.1
20.8	8	21.5	11	0.9
28.8	6	23.1	4	1.2
25.6	7	23.9	7	1.1
38.1	7	27.2	4	1.4
46.1	6	45.6	3	1.0
		Explants from inoculated guinea-pigs		
24.2	9	27.3	6	0.9
14.7	8	33.8	7	0.4
17.4	8	22.9	3	0.8
14.6	6	22.2	3	0.7
18.6	8	42.5	8	0.4
31.9	2	36.7	2	0.9

[a]migration in micrometer units — 2 microns per unit.
[b]n = number of explants.

could be taken of any toxic effect the antigen might have for cells migrating from explants of normal guinea-pigs.

From the migration indices shown in the table mean values for both the normal ($\overline{X}_1$) and sensitive ($\overline{X}_2$) explants were calculated. The statistical significance of the difference between the mean values of the migration indices for samples of this particular size was calculated according to Student's t-test. The following equation was used:

$$t = (\overline{X}_1 - \overline{X}_2) \sqrt{\frac{(N_1 + N_2 - 2)\, N_1 \cdot N_2}{(N_1 + N_2)\, [\Sigma\, (X_1 - \overline{X}_1)^2 + \Sigma\, (X_2 - \overline{X}_2)^2]}}$$

where:

X_1 = individual migration indices of normal explants
X_2 = individual migration indices of sensitive explants
$\overline{X}_1$ = mean of normal migration indices (1.1)
$\overline{X}_2$ = mean of sensitive migration indices (0.7)
N_1 = number of spleens of normal animals (6)
N_2 = number of spleens of sensitive animals (6)

When the values listed are substituted for symbols in the formula, t is calculated as 3.7. The p value is <0.01.

This analysis shows that the differences of migration indices observed between sensitive and normal explants are statistically significant. Therefore it must be concluded that the low migration indices of the sensitive explants resulted from inhibition of cell migration by antigen. In the absence of antigen there was no significant difference in cell migration from the spleens of normal and infected animals. In addition there was no significant effect of the antigen on cell migration from explants of normal animals.

DISCUSSION

Guinea-pigs, after inoculation of *M. lepraemurium,* seem to develop delayed-type hypersensitivity not only to lepraemurium antigen but also to other mycobacterial antigens. This observation tends to confirm the well-known concept of cross-reactivity among mycobacterial antigens. The notion of phylogenetic relationship of all mycobacteria rests in part on cross-reactivity obtained with their proteins in infected hosts. This subject has been reviewed by Xalabarder ([36]). There is not much reason to believe that the results of antigenic analysis depending on formation of visible precipitates must concur with those based on delayed-type cutaneous reactivity of infected hosts. Precipitate formation is limited to the presence of interacting molecules at optimum proportions. Delayed-type sensitivity, on the other hand, is independent of precipitating antibody, and cutaneous responses can be elicited with any amount of antigen greater than an individually variable minimum. The mycobacterial antigens in the present study were protoplasmic fractions of acid-fast bacilli disrupted by mechanical means. Larson *et al.* ([13]) have shown that this fraction, unlike cell-walls, does not produce lesions in the skin of normal animals. This also was our observation, except in some instances where phlei antigen caused a papular lesion in the skin of normal guinea-pigs. These papules appeared soon after injection of antigen and did not exceed 3-5 mm. in diameter. They were never found surrounded by an infiltrated, erythematous zone. These lesions were sufficiently distinct from hypersensitivity reactions to preclude confusion. Our experiments show that living mononuclear cells from peritoneal exudates and splenic cells of guinea-pigs inoculated with *M. lepraemurium* can transfer to normal recipients sensitivity to mycobacterial antigens.

Recently Najarian and Feldman ([18]) accomplished transfer of tuberculin sensitivity with labeled lymphoid cells. They found that the transferred cells accumulated in great numbers at the skin-test site of the recipients. They also observed this in transfer of contact sensitivity to dinitrofluorobenzene ([19]). On the other hand, transferred antihomograft reactivity does not seem to require the presence of transferred cells at the reaction site ([20, 21]). In view of these findings, Najarian and Feldman concluded that tuberculin type sensitivity and homograft reaction were accomplished by different immunologic mechanisms. This may lead one to believe that intact donor cells are indispensable for passive transfer of the delayed type of sensitivity of the infectious and contact type. Nevertheless, some workers have claimed successful transfer of these sensitivities with cell-free extracts ([11, 15, 17]).

As stated before, the toxic effect of antigen on host cells is considered a special attribute of infectious-type hypersensitivity. It has been observed by many investigators ([1, 8, 10, 22, 24, 29, 33]). It should be pointed out, however, that some workers failed to obtain evidence of such an occurrence ([2, 5, 16]). The reasons for this discrepancy remain

unknown. The present experiments show that phlei antigens inhibit cell migration from splenic explants of guinea-pigs inoculated with *M. lepraemurium.* Thornsberry and associates ([32]), studying the effect of culture filtrates of heterologous microorganisms on the migration of leucocytes from tuberculous guinea-pigs, found culture filtrates of *M. phlei* ineffective. It is not known what effect phlei antigen in a concentration as high as that used in our experiments would have on the migration of leucocytes of tuberculous guinea-pigs.

Because of the lack of knowledge in the area of hypersensitivity in leprosy it is planned to extend investigation of the effect of mycobacterial antigen to blood buffy coat explants of patients with various types and reaction states of leprosy.

SUMMARY

Guinea-pigs, after inoculation with *M. lepraemurium,* develop delayed-type hypersensitivity against antigens of the same and other mycobacterial species. Typical, delayed-type skin responses could be elicited with the soluble fraction of *M. lepraemurium, M. phlei,* and *M. bovis* (BCG) prepared by treatment in a sonic oscillator, and with PPD. Reactions to soluble fractions of *M. species* 607 occurred with less regularity. Skin reactions were not obtained after use of a soluble antigen of *M. leprae.* The failure might have been due to low concentration of lepra antigen in this particular preparation.

Delayed-type skin reactions to phlei and BCG antigen could be elicited in normal guinea-pigs 48 hours after intraperitoneal injection of viable mononuclear cells from peritoneal exudations in guinea-pigs hypersensitive to these antigens as a result of previous inoculation with *M. lepraemurium.* Their splenic cells also were capable of transferring this type of hypersensitivity to normal recipient guinea-pigs.

Cell migration from splenic explants of guinea-pigs inoculated with *M. lepraemurium* was significantly inhibited in the presence of the phlei antigen.

RESUMEN

Los cobayos después de la inoculación con *M. lepraemurium* desarrollan un tipo de hipersensibilidad diferida contra los antígenos de las mismas y otras especies de microbacterias. Típicas respuestas de la piel de tipo diferida pueden ser incitadas con la fracción soluble del *M. lepraemurium, M. phlei* y *M. bovis* (BCG) preparados con el tratamiento por un oscilador sónico y con P.P.D. Las reacciones a las fracciones solubles de *M. species* 607 ocurren con menor regularidad. No fueron obtenidas reacciones de la piel después de usar el antígeno soluble del *M. leprae.* El fracaso puede deberse a la baja concentración del antígeno leproso en esta preparación en particular.

Las reacciones diferidas en la piel al phlei y al antígeno BCG, pueden ser inducidas en los cobayos normales 48 horas después de la inyección intraperitoneal de celulas mononucleares viables del exudado peritoneal, en cobayos hipersensitivos a estos antígenos, como un resultado de la inoculacíon previa con *M. lepraemurium.* Sus células esplenicas fueron también capaces de transferir este tipo de hipersensibilidad a cobayos receptores normales.

La migración de células de explantes esplénicos de cobayos inoculados con el *M. lepraemurium* fué significativamente inhibida en la presencia de antígeno phlei.

RÉSUMÉ

Après inoculation avec *M. lepraemurium*, des cobayes développent une hypersensibilité de type retardé aux antigènes de la même espèce ou d'autres espèces mycobactériennes. Des réactions cutanées de type retardé typiques peuvent être provoquées par la fraction soluble de *M. lepraemurium*, de *M. phlei* et de *M. bovis* (BCG) obtenues par passage dans un oscillateur ultra-sonique, ainsi qu'avec le PPD. Des réactions à la fraction soluble de *Mycobactéries* sp. 607 surviennent moins régulièrement.

Aucune réaction cutanée n'a été obtenue avec un antigène soluble de *M. leprae*. L'échec peut avoir été du à la faible concentration d'antigène lépreux dans la préparation en cause. Des réactions cutanées de type retardé à l'antigène de *M. phlei* et au BCG ont pu être obtenues chez des cobayes normaux 48 heures après l'injection intrapéritonéale de cellules mononucléaires viables provenant d'exsudats intra-péritonéaux de cobayes hypersensibles à ces antigènes suite à une inocúlation antérieures avec *M. lepraemurium*. Les cellules splénique de ces cobayes sont également capables de transférer ce type d'hypersensibilité à des cobayes normaux lorsqu'ils les reçoivent.

La migration de cellules splénique explantées de cobayes inoculés avec *M. lepraemurium* a été significativement inhibée par la présence d'antigène phléique.

REFERENCES

1. Aronson, J. D. The specific cytotoxic action of tuberculin in tissue culture. J. Exper. Med. **54** (1931) 387-397.
2. Baldrige, G. D. and Kligman, A. M. The nature of the tuberculin reaction. Failure to demonstrate *in vitro* cytotoxicity of tuberculin for the cells of sensitized animals. American Rev. Tuberc. **63** (1951) 674-678.
3. Chase, M. W. The cellular transfer of cutaneous hypersensitivity to tuberculin. Proc. Soc. Exper. Biol. & Med. **59** (1945) 134-135.
4. Cochrane, R. G. Leprosy in Theory and Practice. Bristol, England, John Wright & Sons, Ltd., 1959, pp. 114-119.
5. Cruickshank, C. N. D. Sensitivity to tuberculin. Nature, London **168** (1951) 206-207.
6. Cummings, M. M., Hoyt, M. and Gottshall, R. Y. Passive transfer of tuberculin sensitivity in the guinea pig. Publ. Hlth. Rep. **62** (1947) 994-997.
7. Cummings, M. M., Patnode, R. A. and Hudgins, P. C. Passive transfer of tuberculin hypersensitivity in guinea pigs using cells disrupted by sonic vibration. American Rev. Tuberc. & Pulm. Dis. **73** (1956) 246-250.
8. Favour, C. B. Lytic effect of bacterial products on lymphocytes of tuberculous animals. Proc. Soc. Exper. Biol. & Med. **65** (1947) 269-272.
9. Fernandez, J. M. M. The early reaction induced by lepromin. Internat. J. Leprosy **8** (1940) 1-14.
10. Heilman, D. H., Feldman, W. H. and Mann, F. C. Specific cytotoxic action of tuberculin; quantitative studies in tissue cultures. American Rev. Tuberc. **50** (1944) 344-356.
11. Jeter, W. S., Tremaine, M. M. and Seebohm, P. M. Passive transfer of delayed hypersensitivity to 2,4-dinitrochlorobenzene in guinea pigs with leukocytic extracts. Proc. Soc. Exper. Biol. & Med. **86** (1954) 251-253.
12. Kirchheimer, W. F. and Weiser, R. S. The tuberculin reaction. I. Passive transfer of tuberculin sensitivity with cells of tuberculous guinea-pigs. Proc. Soc. Exper. Biol. & Med. **66** (1947) 166-170.
13. Larson, C. L., Bell, J. F., List, R. H., Ribi, E. and Wicht, W. C. Symposium on relationship of structure of microorganisms to their immunological properties. II. Host-reactive properties of cell walls and protoplasm from mycobacteria. Bact. Rev. **27** (1963) 341-351.
14. Lawrence, H. S. Cellular transfer of cutaneous hypersensitivity to tuberculin in man. Proc. Soc. Exper. Biol. & Med. **71** (1949) 516-522.

15. Lawrence, H. S. Transfer in humans of delayed skin sensitivity to streptococcal M substance and to tuberculin with disrupted leukocytes. J. Clin. Invest. **34** (1955) 219-230.
16. Marks, J. and James, D. M. The effect of tuberculin on sensitized and normal leukocytes. J. Hyg. **51** (1953) 340-346.
17. Morris, J. A., Aulisio, C. G., Boseman, F. M. and Guinto, R. S. Fluorescent antibody study of the human leprosy bacillus. Bact. Proc., 1961, p. 123.
18. Najarian, J. S. and Feldman, J. D. Passive transfer of tuberculin sensitivity by tritiated thymidine-labeled lymphoid cells. J. Exper. Med. **114** (1961) 779-790.
19. Najarian, J. S. and Feldman, J. D. Passive transfer of contact sensitivity by tritiated thymidine-labeled lymphoid cells. J. Exper. Med. **117** (1963) 775-780.
20. Najarian, J. S. and Feldman, J. D. Passive transfer of transplantation immunity. I. Tritiated lymphoid cells. II. Lymphoid cells in Millipore chambers. J. Exper. Med. **115** (1962) 1083-1093.
21. Najarian, J. S. and Feldman, J. D. Passive transfer of transplantation immunity. III. Inbred guinea pigs. J. Exper. Med. **117** (1963) 449-456.
22. O'Neil, E. F. and Favour, C. B. Tissue culture analysis of tuberculin hypersensitivity in man. American Rev. Tuberc. **72** (1955) 577-600.
23. Osgood, E. E. and Ashworth, C. M. Atlas of Hematology. San Francisco, Calif., J. W. Stacey, Inc., 1937, pp. 206-207.
24. Paas, C. M., Flick, J. A., Kapral, F. A. and Rudd, J. H. On the persistence of tuberculin sensitivity in guinea pigs made sensitive passively. American Rev. Resp. Dis. **83** (1961) 372-380.
25. Raffel, S. The mechanism involved in acquired immunity to tuberculosis. CIBA Foundation Symposium on Experimental Tuberculosis. Boston, Mass., Little, Brown & Co., 1955, pp. 261-279.
26. [Rio Congress] Technical resolutions. Report of panel on lepra reaction. VIIIth Internat. Congr. Leprol., Rio de Janeiro, 1963. Internat. J. Leprosy **31** (1963) 480-482.
27. [Rio Congress] Technical resolutions. Report of the round table on borderline and indeterminate leprosy. VIIIth Internat. Congr. Leprol., Rio de Janeiro, 1963. Internat. J. Leprosy **31** (1963) 478-480.
28. Rich, A. R. The Pathogenesis of Tuberculosis, 2nd ed. Springfield, Ill., Charles C. Thomas, 1951.
29. Rich, A. R. and Lewis, M. R. The nature of allergy in tuberculosis as revealed by tissue culture studies. Bull. Johns Hopkins Hosp. **50** (1932) 115-131.
30. Sanford, J. P., Favour, C. B. and Lindsey, E. Effect of tuberculin upon oxygen utilization of blood and of splenic tissue from tuberculous and normal guinea pigs. American Rev. Tuberc. & Pulm. Dis. **73** (1956) 581-585.
31. Shepard, C .C. The nasal excretion of *Mycobacterium leprae* in leprosy. Internat. J. Leprosy **30** (1962) 10-18.
32. Thornsberry, C., Gerlach, E. H. and Sherago, M. The effect of culture filtrates of heterologous microorganisms on the *in vitro* migration of leukocytes from tuberculous guinea pigs. Bact. Proc., 1963, p. 60.
33. Waksman, B. H. Studies on cellular lysis in tuberculin sensitivity. American Rev. Tuberc. **68** (1953) 746-759.
34. Wallace, J. H. Leukocytic transfer of delayed sensitivity to normal guinea pigs from rats infected with *Mycobactèrium lepraemurium*. J. Immunol. **80** (1958) 362-366.
35. Wesslen, T. Passive transfer of tuberculin hypersensitivity by viable lymphocytes from the thoracic duct. Acta Tuberc. scandinav. **26** (1952) 38-53.
36. Xalabarder, C. The so-called problem of unclassified mycobacteria, a review. American Rev. Resp. Dis. **83** (1961) 1-15.