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Immune Response to *Mycobacterium tuberculosis* in Rats

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After intravenous injection into rats, both the attenuated strain R1Rv and the virulent strain H37Rv of *Mycobacterium tuberculosis* grow in the liver and spleen. However, the infected rats mount a specific immune response with great rapidity, giving a false impression of natural resistance to the tubercle bacillus. Adoptive immunity to tuberculosis was achieved by transferring thoracic duct cells from immunized donors to normal syngeneic recipients. The transferred immunity was vested in a population of lymphocytes uncontaminated with macrophages. The adoptive immunity was effectively expressed against both attenuated and virulent tubercle bacilli, and it was shown to be immunologically specific. Lymphocytes which conferred immunity to tuberculosis were not protective against *Listeria monocytogenes* infection, and vice versa. Immunity could not be transferred with either normal thoracic duct lymphocytes (TDL), heat-killed sensitized TDL, or serum from specifically immunized donors. The ability of TDL from BCG-immunized donors to confer immunity was maintained at an unimpaired level for at least 3 months after immunization.

For many years rats have been considered highly resistant to tuberculosis and, by implication, unsuitable for studies in experimental tuberculosis. Early workers had established that rats survived infection with doses of virulent tubercle bacilli many times higher than those which kill guinea pigs (8, 14, 22). These investigators were unable to elicit tuberculin hypersensitivity in tuberculous rats. Subsequently, Wessels (28) demonstrated the growth of virulent tubercle bacilli in the organs of rats, which also developed delayed hypersensitivity to tuberculin. These results were confirmed by Gray et al. (11), who suggested that rats are no less susceptible to tuberculosis than are some strains of mice. Since then, delayed hypersensitivity to other antigens has also been induced in rats (5).

Despite the latter findings, rats have been little used in tuberculosis research and, in a recent analysis of experimental models for the study of immunity in tuberculosis, rats were not even considered (30). Yet rats have potential advantages over other species in the study of immune mechanisms: the physiology of their lymphocytes has been characterized most extensively and the thoracic duct which discharges relatively pure populations of lymphocytes can be cannulated with comparative ease (4, 7, 12). It was therefore decided to initiate a study of tuberculosis in rats to see whether these advantages could be exploited to characterize the cells responsible for antibacterial immunity.

MATERIALS AND METHODS

Animals. Female (Lewis × BN)F_1 rats were used. Cell donors were 8 to 12 weeks old, 150 to 200 g in weight. Recipient rats were 5 weeks old and 80 to 100 g in weight.

Cultures. Strains of mycobacteria were obtained from the Trudeau Mycobacterial Culture Collection, Trudeau Institute, Saranac Lake, N.Y. Two attenuated strains, BCG Copenhagen (TMC 1010) and R1Rv (TMC 205), and one virulent strain, H37Rv (TMC 102), were used. All mycobacterial cultures were maintained by serial passage in Middlebrook 7H9 broth (Difco Laboratories, Detroit, Mich.). Cultures were used after 5 to 8 days of incubation at 37 C.

A virulent strain of *Listeria monocytogenes* was stored in liquid nitrogen (15), from which it was recovered as required.

Immunization. A culture of mycobacteria was washed four times in saline containing 0.05% Tween 80 (polyethylene sorbitan monooleate; BBL) by centrifugation at 5650 × *g* for 20 min. The culture was reconstituted to its original volume in Tween-saline and briefly ultrasonicated to disperse clumps. Rats were immunized by injecting 0.1-ml samples of bacterial suspension into eight sites distributed over the

hindquarters: into each footpad, subcutaneously into the medial aspect of each leg, subcutaneously into each groin. There was a single subcutaneous injection into the base of the tail and a single intraperitoneal injection. The immunization technique was based upon that described by Delorme and others (3).

L. monocytogenes was diluted to a density of 2.5×10^7 viable bacteria/ml and 0.1-ml samples were injected into each footpad, a total animal dose of 5×10^6 organisms. The following day a fresh *Listeria* suspension of 2.5×10^7 viable bacteria/ml was made, and 0.1-ml aliquots were injected subcutaneously into the base of the tail and at three lower abdominal sites, a total animal dose of 10^7 organisms.

Challenge. For growth curve data or challenge of cell recipients, an unwashed mycobacterial culture was briefly sonicated, and a total bacterial count was made in a Petroff-Hausser counting chamber. The culture was diluted, as necessary, in sterile saline, and 1-ml samples were injected intravenously. *L. monocytogenes* cultures were appropriately diluted in saline and 1 ml was injected intravenously into each rat.

Viable counts. Viable counts of bacteria were made from inoculum suspensions and from liver and spleen homogenates. Mycobacteria were grown on Middlebrook 7H10 agar plates (Difco), which were incubated at 37 C for 3 weeks. *L. monocytogenes* was grown on tryptic-soy agar plates (BBL, Cockeysville, Md.), which were incubated at 37 C overnight. Five rats from each experimental group were killed at each time point, and the geometric mean viable count per group was determined. Analysis of variance was used to evaluate the experimental data.

Cell transfer. Lymphocytes obtained from the thoracic duct of donor rats were collected during the first 24 h of lymph drainage. The cells were counted, centrifuged from the lymph, and resuspended at a cell density of 10^8/ml in Hanks basal salt solution containing 1% fetal calf serum. In all experiments, the thoracic duct lymphocytes (TDL) were injected intravenously.

Immune serum. Rats were immunized with BCG as described above. On the eighth day of infection the rats were bled from the throat, and the serum was allowed to separate. The serum was sterilized by membrane filtration and stored at -70 C. The serum was not inactivated by heat before use.

RESULTS

Growth of R1Rv in livers and spleens of normal rats. Rats were injected intravenously with 1-ml samples of a serial 50-fold dilution of R1Rv culture, and viable counts of R1Rv were made from liver and spleen homogenates at predetermined intervals after infection. The geometric mean viable counts were estimated, and it is clear that R1Rv grew in both the liver and spleen (Fig. 1). The initial increase of R1Rv was inversely related to the inoculum size, and the subsequent decline in the bacterial populations was initiated more rapidly in rats infected with the large inocula than with the smallest inoculum (15). Over a 14-day period, the growth of the smallest inoculum of R1Rv was substantial: 1.25 and 2.07 $\log_{10}$ units in the liver and spleen, respectively.

Growth of H37Rv and R1Rv in the organs of immunized rats. Rats were either infected with 10^5 viable R1Rv intravenously, or were set aside as controls. Four weeks later, groups of normal and R1Rv-infected rats were challenged intravenously with approximately 10^5 R1Rv or 10^5 H37Rv. The challenge inoculum of H37Rv was slightly higher than that of R1Rv (Fig. 2). Both organisms grew to a similar extent in the organs of normal rats, whereas their growth was completely inhibited in immunized subjects. H37Rv, however, was eliminated more slowly than was R1Rv. There are reasons for thinking that the higher spleen counts observed in reinfected rats on day zero was related to residual R1Rv from the primary immunizing infection, but this was not formally established.

Development of immunity to tuberculosis. The development of resistance to tuberculosis was measured in terms of the protective immunity transferred by TDL obtained at various intervals after infection. Prospective donors were immunized with either BCG or R1Rv by the subcutaneous-intraperitoneal technique at

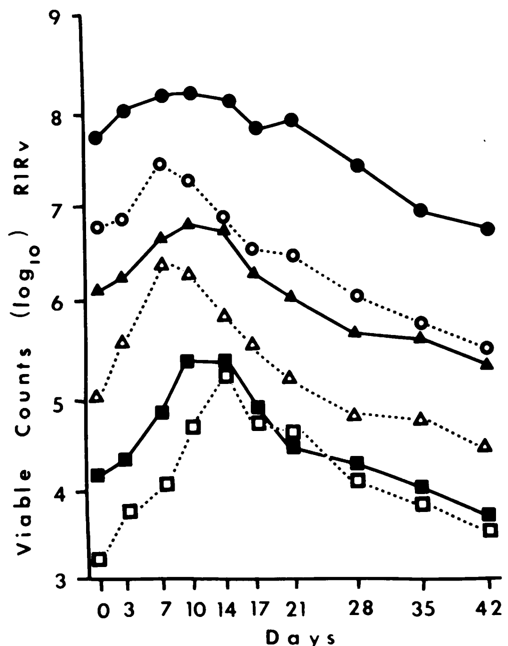

FIG. 1. *Growth of three inocula of R1Rv in the liver and spleen of rats. Closed symbols, liver data; open symbols, spleen data. Infecting inocula of R1Rv per rat: 5×10^7, (●), 1.5×10^6, (▲); and 2.5×10^4, (■).*

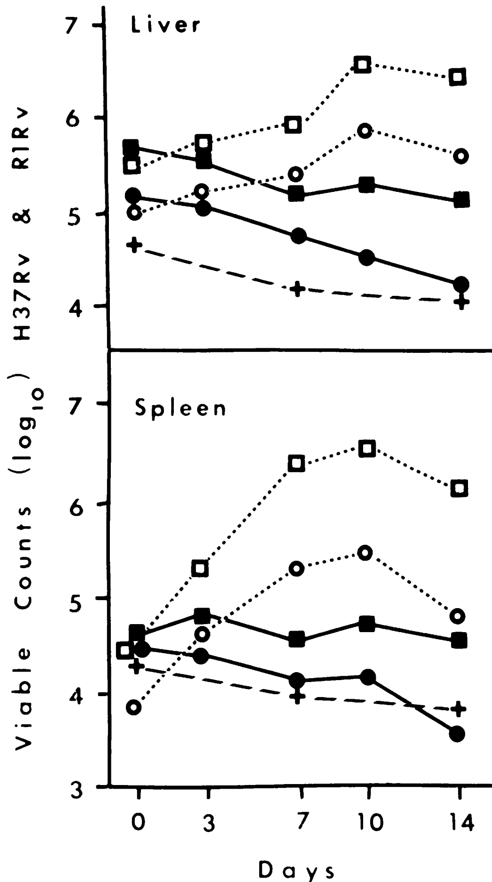

FIG. 2. *Growth of R1Rv and H37Rv in normal and actively immunized rats. Open symbols, normal rats; closed symbols, R1Rv-immunized rats. R1Rv challenge,* ○; *H37Rv challenge,* □; *residual population of immunizing R1Rv inoculum,* ✚.

10, 8, 6, or 4 days before incannulation. In one experiment, TDL were also obtained from normal donors. Recipient rats were challenged with approximately 10^4 R1Rv and divided into groups which were given 2×10^8 TDL/100 g of body weight. A control group received no cells. Viable counts of R1Rv in liver and spleen were made 10 days after challenge (Table 1). It is clear that normal cells in no way inhibited the growth of R1Rv and that cells from immunized animals had little, if any, effect upon the growth of R1Rv in the liver. Such differences from the control rats as were observed were not statistically significant. By contrast, cells obtained as early as 4 days after immunization with R1Rv conferred a significant degree of immunity in the spleen ($P < 0.05$). In the case of R1Rv- and BCG-immunized donors, the level of protection transferred by a standard inoculum of TDL increased to a maximum on days 6 and 8, respectively. In both experiments, the increase in protection between days 4 and 8 was highly significant ($P < 0.01$). The inability of adoptively immunized rats to express antimycobacterial immunity in the liver as compared with the spleen has been a feature of all experiments.

An attempt was made to passively immunize rats with serum or dead cells from BCG-infected donors. Serum and TDL were obtained from rats immunized 8 days earlier with 10^8 viable BCG. A sample of TDL was incubated in a water bath at 60 C for 20 min. The viability of the cells was 97%, preincubation, and 0.5%, postincubation. Recipient rats challenged with 5×10^4 R1Rv were given either 2×10^8 viable TDL, 2×10^8 dead TDL, or immune serum. A control group of recipients received no treatment. The cells were given in a single intravenous injection, whereas serum treatment consisted of a 1-ml intravenous injection on days 0, 2, 4, 6, 8, 10, and 12 relative to the challenge infection, a total of 7 ml of serum per rat. All recipients were killed 14 days after challenge, and viable counts of R1Rv were made from each spleen (Table 2). Neither immune serum nor dead TDL influenced the growth of R1Rv. By contrast, the growth of R1Rv was greatly reduced in rats receiving viable TDL.

Duration of immunity in BCG-immunized rats. Groups of rats immunized subcutaneously-intraperitoneally with living BCG were incannulated 1, 2, 3, 4, 6, 8, or 12 weeks after immunization. Cells obtained from these animals were pooled with those from donors in the same group and 2×10^8/100 g were transferred intravenously into recipient rats which had been challenged with approximately 2.5×10^4 R1Rv. Viable counts of R1Rv were made from the livers and spleens of these adoptively immunized subjects and nonimmunized controls. Initially, counts were made 3 h and 3, 7, 10, and 14 days after challenge. In the latter part of the experiment, only 3-h and 14-day counts were obtained.

At each time point there was little difference between the adoptively immunized and control groups with respect to viable counts in the liver; but when all the 14-day postchallenge viable counts were pooled, those obtained from cell recipients were significantly lower ($P < 0.01$) than those from controls. As in the preceding experiments, cell transfer resulted in a substantial degree of protection in the spleen (Fig. 3 and Table 3). During the 4-week period that growth curves were obtained, the general pattern in adoptively immunized rats was similar. The uniformity of the adoptive immune response

TABLE 1. *Development of the ability to confer adoptive immunity to tuberculosis*[a]

Samples tested	Interval between immunization and thoracic duct cannulation (days)	BCG			RlRv		
		Immunizing dose (× 10^7)	Viable counts of RlRv ($\log_{10}$)		Immunizing dose (× 10^7)	Viable counts of RlRv ($\log_{10}$)	
			Liver	Spleen		Liver	Spleen
Immunized rats	10	6.6	5.01	3.56	1.7	4.61	3.29
	8	5.5	4.94	3.48	2.5	4.73	3.26
	6	4.3	5.11	3.84	6.5	4.69	3.26
	4	3.6	5.21	4.23	4.0	4.90	4.16
Normal donors		Nil	5.44	5.05			
Control (no cell transfer)			5.27	4.89		5.06	4.66

[a] Rats were immunized with BCG or RlRv in the dosages indicated.

TABLE 2. *Effect of immune serum, viable thoracic duct cells, and dead thoracic duct cells on the growth of R1Rv*

Treatment	Viable counts RlRv ($\log_{10}$) in spleen
None	4.98
Immune serum	5.05
Dead thoracic duct cells	5.12
Viable thoracic duct cells	3.55

TABLE 3. *Adoptive immunity conferred by thoracic duct cells obtained at intervals following immunization*

Interval between immunization and cannulation (weeks)	Difference in spleen viable counts of RlRv ($\log_{10}$) between normal and adoptively immunized rats on day			
	3	7	10	14
1	0.20	0.69	1.17	1.38
2	0.22	0.84	0.89	1.39
3	-0.17	0.41	0.64	1.01
4	-0.24	0.43	1.18	1.10
6				1.05
8				1.26
12				1.28

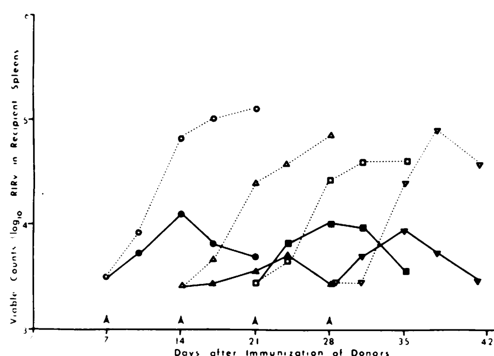

FIG. 3. *Growth of R1Rv in the spleens of normal and adoptively immunized rats. Thoracic duct lymphocytes were obtained from BCG-infected donors either 7 (●), 14 (▲), 21 (■), or 28 (▼) days after immunization. Open symbols represent corresponding groups of normal control rats.*

was indicated by the 14-day postchallenge protection values (Table 3, last column), estimated by subtracting the mean viable counts ($\log_{10}$) of immunized rats from those of normal rats. The degree of protection ranged from 1.01 to 1.39 log U, a 10- to 25-fold decrease of viable counts compared with controls, during the 3-month period of examination. Maximum immunity was conferred by cells obtained 1 to 2 weeks after immunization, and the ability to confer immunity remained substantially unchanged thereafter.

The results in Fig. 3 show another interesting and consistent feature, namely that the adoptive immunity conveyed by TDL did not completely inhibit the growth of the challenge organism. In all of the adoptively immunized animals, R1Rv grew in the spleen for as long as 7 days, after which the viable counts declined. By contrast, R1Rv grew for at least 10 days, and usually for 14 days in nonimmunized rats. The difference between the viable counts in the spleens of adoptively immunized rats and their respective controls was insignificant 3 days after challenge, but was highly significant from 7 days onward.

Effect of cell numbers on adoptive immunity. Donor rats were immunized with 3.4 × 10^8 BCG by the subcutaneous-intraperitoneal route, and thoracic duct cannulations were performed 8 days later. Lymphocytes obtained from these animals were transferred intravenously into recipients which had been challenged by the same route with 1.7 × 10^4 R1Rv. Groups of five recipient rats received intravenously either 10^9, 2 × 10^8, or 4 × 10^7 cells/100 g

of body weight; a fourth group received no cells. Viable counts of R1Rv in the livers and spleens were made 10 days after challenge. Increasing the number of cells fivefold from 2×10^8 to 10^9 did not increase the degree of protection in the spleen, but a fivefold reduction in the number of transferred cells, from 2×10^8 to 4×10^7, reduced the level of protection substantially (Table 4). Even so, the immunity conferred by 4×10^7 cells was highly significant ($P < 0.01$). As noted previously, even the largest cell dose conferred little protection on the liver.

Adoptive immunity against a virulent challenge. TDL obtained from donor rats on the eighth day of an immunizing BCG infection were transferred into recipient rats. The cells, in a dose equal to 2×10^8/100 g, were injected 1 h after the recipients had been infected with either 3×10^4 R1Rv or 3×10^4 H37Rv. Viable counts of mycobacteria in the livers and spleens of these adoptively immunized animals and groups of nonimmunized controls were made 14 days after challenge (Table 5). The counts of H37Rv were higher than those of R1Rv in livers and spleens of both normal and adoptively immunized subjects, a reflection of the virulence of the former strain. Adoptively immunized rats inhibited the growth of both organisms to a similar extent: the levels of protection in the spleens were 1.39 and 1.21 log U for R1Rv and H37Rv, respectively. Once again, protection in the liver was minimal.

Specificity of the adoptive immune response. The specificity of the immunity transferred with TDL was tested reciprocally: cells from *Listeria* and BCG immunized rats being measured for protective activity against both R1Rv and *L. monocytogenes*.

TABLE 4. *Effect of cell dose on adoptive immune response to tuberculosis*

No. of thoracic duct lymphocytes transferred	Viable counts of R1Rv ($\log_{10}$)	
	Liver	Spleen
0	4.04	3.84
4×10^7	3.91	3.05
2×10^8	3.64	2.34
10^9	3.75	2.39

TABLE 5. *Adoptive immunity against virulent challenge*

No. of thoracic duct lymphocytes tested	Viable counts ($\log_{10}$) 14 days after challenge			
	Liver		Spleen	
	R1Rv	H37Rv	R1Rv	H37Rv
0	5.24	5.69	4.67	5.63
2×10^8	4.81	5.49	3.28	4.42

TABLE 6. *Specificity of the immunity conferred with thoracic duct lymphocytes from listeria-immunized rats*

No. of thoracic duct lymphocytes tested	Viable counts ($\log_{10}$)			
	L. Monocytogenes		R1Rv	
	Liver	Spleen	Liver	Spleen
0	5.80	6.54	5.81	5.34
2×10^8	3.00	2.90	5.95	5.53

TDL were collected 6 days after donor rats had been immunized with *L. monocytogenes*, and 2×10^8 TDL were injected into recipients who had been challenged intravenously with either 10^6 *L. monocytogenes* or 7×10^4 R1Rv (Table 6). Appropriate control rats did not receive cells. Viable counts of *L. monocytogenes* and R1Rv were made from the liver and spleen 48 h and 14 days after challenge, respectively. The immunity conferred adoptively was specific to *L. monocytogenes*, there being no evidence of cross-protection against *M. tuberculosis* R1Rv.

In looking at the opposing situation, account was taken of the fact that BCG provokes a substantial level of nonspecific, macrophage-mediated resistance (2). It is known that nonspecific resistance is caused by the action of antigen upon specifically sensitized lymphocytes (18). To provoke this reaction, the dose of R1Rv used to challenge the adoptively immunized rats and their controls was deliberately increased to 5×10^6.

Groups of recipient rats were infected intravenously with either 5×10^4 R1Rv, 10^6 *L. monocytogenes*, or both organisms. Half of the rats in each group were given TDL obtained on the eighth day of a BCG infection; the remaining rats received no cells. Protection conferred by TDL was specific (Table 7): the recipients of TDL inhibited the growth of R1Rv (rows 1 and 2), but not the growth of *L. monocytogenes* (rows 3 and 4). In addition, the experiment reveals that rats whch had been adoptively sensitized to R1Rv also developed a significant measure ($P < 0.01$) of nonspecific resistance to *L. monocytogenes* only when the cell recipients were challenged simultaneously with R1Rv (rows 5 and 6).

DISCUSSION

The natural resistance of the rat to infection with tubercle bacilli is an established part of the folklore pertaining to experimental tuberculosis. Like much folklore, this notion rests on

TABLE 7. *Specificity of the immunity conferred by thoracic duct lymphocytes from BCG-immunized rats*

Challenge infection		Thoracic duct lymphocyte transfer	Viable count (log_{10})			
			R1Rv		*L. monocytogenes*	
R1Rv	*L. monocytogenes*		Liver	Spleen	Liver	Spleen
+			7.09	6.61		
+		+	6.90	5.62		
	+				5.22	5.80
+	+				5.03	6.08
	+	+			5.88	6.46
+	+	+			5.50	5.72

insubstantial evidence, derived mainly from the study of Ornstein and Steinbach (22), who infected rats with a large dose of virulent tubercle bacilli which failed to kill the host, render it sensitive to tuberculin, or produce the typical lesions of tuberculosis. Of these findings, only the resistance of rats to the lethal effects of tuberculosis has been adequately substantiated. Many investigators have described the formation of noncaseating tubercles in rats (8, 14, 16), and Hehre and Freund (14) observed systemic tuberculin sensitivity but no dermal sensitivity. Subsequently, Wessels (29, 30) found that tubercle bacilli could grow in the organs of rats and cause delayed-type tuberculin hypersensitivity. These findings have been amply confirmed more recently (10, 11). Despite this evidence, the original notion persists that rats are naturally resistant to tuberculosis and hence unsuitable for the study of immunity in this infection.

In the present study it was shown that the virulent strain H37Rv and the attenuated strain R1Rv can grow in the liver and spleen of intravenously infected rats. Although data have not been presented, the even more attenuated strain BCG multiplied in the same organs. These observations have been confirmed in Lewis rats and members of the (Lewis × DA)F_1 cross, supporting the proposition of Gray (10) that the growth of tubercle bacilli in rats is comparable with that in mice.

After infection with 10^7 to 10^8 BCG or R1Rv, rats rapidly develop a state of acquired immunity to tuberculosis. As early as 4 days after infection, TDL were capable of conferring specific resistance upon normal syngeneic recipients, and maximum immunity was conveyed by cells obtained 6 to 10 days after infection (Table 1). As a consequence of the extremely rapid production of cells with protective activity, large inocula of tubercle bacilli do not grow, thus giving a spurious appearance of natural resistance in this host species (15). In truth, the apparent natural resistance of the rat is due to rapid induction of acquired immunity, as inferred by previous investigators (23, 24).

Adoptive immunity to tuberculosis has been achieved in rabbits (6, 17), guinea pigs (26), and mice (20, 25). With the exception of Sever (25), all these investigators used randomly bred animals in which the inevitable reactions of histocompatibility would raise resistance nonspecifically (1) and call in question the significance of the effects observed. In addition, mixed populations of lymphoid cells (lymph node, spleen, or peritoneal exudate cells) have been used in all previous studies. These cell populations included macrophages, which some investigators construed to be the cell type predominantly involved in the transfer of immunity (6, 20, 25). It is significant, therefore, that in the present investigation immunity was transferred by lymphocytes obtained from the thoracic duct. Immunity acquired in this manner was clearly mediated by living donor cells, for it was related to the number transferred and the level of resistance in the donors. Moreover, heat-killed cells or serum from immune donors conveyed no protection whatsoever.

Cellular events underlying the development of immunity to the tubercle bacillus cannot readily be analyzed in the infected animal because the response to infection involves not only the production of specifically sensitized lymphocytes but also secondary changes in macrophages, the cells through which resistance to infection is ultimately expressed. It is to be noted in this connection that the macrophages of tuberculous animals acquire an enhanced microbicidal capability which is nonspecific, because it is expressed against a variety of bacterial cell targets; but the participation of specifically sensitized lymphocytes is an essential requirement for the development of activated macrophages (19). This was again illustrated in the present study by the specificity of the immunity transfered with lymphocytes. TDL from *Listeria*-infected rats protected against *L. monocytogenes* but not against

R1Rv, and vice versa.

The rats which were infected with R1Rv and superinfected with *L. monocytogenes* are of particular interest. Animals which received only the TDL from BCG-sensitized donors or were infected with R1Rv without receiving cells were fully susceptible to *L. monocytogenes;* but those which received TDL from BCG-immunized rats and were also challenged with R1Rv showed a small but significant level of resistance to *L. monocytogenes*. This phenomenon is analogous to the rapid recall of nonspecific resistance which is observed when BCG-immunized mice are reinfected with the homologous organism (2). It occurs also in mice challenged with BCG after adoptive sensitization with spleen cells from BCG-immunized donors (18).

A constant but unexplained feature of this investigation was the difference between actively and adoptively immunized rats in the expression of immunity in the liver and spleen. Immunity to reinfection was expressed in both organs, whereas the immunity transferred by TDL from specifically immunized donors was expressed only in the spleen (Table 4 and Fig. 2). The apparent defect in the expression of antimicrobial resistance in the livers of adoptively immunized rats is much less apparent in rats adoptively immunized against listeria and is not observed in animals actively infected with BCG (Fig. 2). Immunity in the latter represents the summation of two effector mechanisms. One is mediated by macrophages, which are already activated at the time of reinfection, and a second is mediated by specifically sensitized lymphocytes which respond to the reinfecting organisms. Because Kupffer cells in the liver become integrated into developing tubercles (R. J. North, unpublished data), the tubercles formed in reinfected animals would be composed from the outset of highly activated phagocytes, whereas those formed in adoptively immunized animals under the influence of specifically sensitized lymphocytes would be constructed of circulating monocytes and nonactivated Kupffer cells (21). Clearly, the reinfected animals would have an advantage.

Although this explanation may account for the difference between active and adoptive immunity as expressed in the liver, it does not explain why a difference should exist between spleen and liver in the adoptively immunized rat. The failure to express adoptive immunity is not peculiar to the liver, however, for an even greater defect has been found in the ability of the adoptively immunized mouse to defend the lung against an aerogenic infection with *L. monocytogenes* (27). A possible reason for this phenomenon is the sequestration in the spleen and lymph nodes of intravenously injected lymphocytes obtained either from normal (9, 13) or specifically sensitized donors (Sutton and Mackaness, unpublished data). It may be, therefore, that the spleen benefits more from an infusion of specifically sensitive lymphocytes for purely numerical reasons. This hypothesis fails to explain the excellent protection expressed in the livers of animals adoptively immunized against *L. monocytogenes* (Table 6), and raises an alternative possibility based upon a difference in the composition of the specifically sensitized lymphocyte populations used to transfer resistance to listeria and tuberculosis. It could be that TDL are a selected population which is not representative of the cells available for the defences of the intact animal. This question has been investigated in a study dealing with the properties of the lymphocytes which mediate host resistance to the tubercle bacillus (M. J. Lefford, D. D. McGregor, and G. B. Mackaness, Immunology, in press).

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