INFECTION AND IMMUNITY, Mar. 1981, p. 1023-1033
0019-9567/81/031023-11$02.00/0

Vol. 31, No. 3

Induction and Suppression of Cross-Reactive Antituberculosis Immunity After *Mycobacterium lepraemurium* Infection of Mice

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Mice immunized with 10^8 live *Mycobacterium lepraemurium* in the footpad showed increased resistance to infection with BCG or *M. tuberculosis* R1Rv. This resistance could be transferred adoptively with lymphoid cells, signifying that the immunity was cross-reactive rather than nonspecific. Adoptive cross-reactive immunity to *M. tuberculosis* was also conferred by spleen cells from mice immunized with large doses of living or dead *M. lepraemurium* intravenously, a route of immunization that suppresses the induction of cell-mediated immunity to that organism. The presence of specific suppressor activity was sought in mice immunized intravenously with *M. lepraemurium*. It was found that mice preimmunized intravenously with living or dead *M. lepraemurium* and then infected with BCG did not confer levels of adoptive antituberculosis immunity as high as those conferred by mice immunized with BCG alone. Similarly, a mixture of BCG-sensitized and *M. lepraemurium*-sensitized cells did not convey as much immunity as BCG-sensitized cells alone, signifying suppression of the effector lymphocytes.

In an earlier study, it was shown that immunization with living BCG favorably modified the course of subcutaneous, but not intravenous (i.v.), infection with *Mycobacterium lepraemurium*. Evidence was presented that BCG-induced immunity against *M. lepraemurium* was cross-reactive rather than nonspecific. It might be expected that such cross-reactive immunity would be reciprocal, in which case immunization with *M. lepraemurium* would induce immunity against the *M. tuberculosis-M. bovis* complex, and a preliminary experiment appeared to support this hypothesis (14).

The significance of such cross-reactive immunity is that it provides a tool for analyzing the regulation of the immune response to *M. lepraemurium* infection. That infection is difficult to study because the organism is not quantitatively cultivable in vitro. Consequently, protective immunity to *M. lepraemurium* is measured by counting the total bacteria in stained smears of tissue homogenates. Total bacterial counts include both living and dead mycobacteria, a problem that is exacerbated by the fact that mycobacteria remain stainable for long periods after death. Living mycobacteria may be differentiated from dead mycobacteria in stained preparations by morphological criteria (20, 21), but the distinction may be difficult, subjective, and open to other interpretations (6).

These problems might be circumvented by using an easily cultivable cross-reactive organism, such as *M. bovis* or *M. tuberculosis*, to measure the specific immunity generated by *M. lepraemurium* infection. This paper reports the results of an exploration of this possibility.

MATERIALS AND METHODS

Animals. Inbred female mice of the $CB6F_1$ (BALB/c × C57BL/6) strain were used.

Mycobacteria. The cultivable organisms *M. bovis* BCG Pasteur (TMC 1011) and *M. tuberculosis* R1Rv (TMC 205) were obtained from the Mycobacterial Culture Collection of Trudeau Institute Inc., Saranac Lake, N.Y. Cultures were grown in roller bottles at 37°C in Proskauer and Beck medium supplemented with 2% glycerol and 0.1% Tween 80. Fully grown cultures were stored at −70°C, and their viability was determined. Before use, cultures were thawed and appropriately diluted in sterile saline to provide the desired inocula of viable bacilli.

M. lepraemurium was passaged in mice from whose livers and spleens the organisms were purified by the nonenzymatic separation procedure of Draper (7). These bacteria were stored at −70°C at a known density and recovered as required. Heat-killed (HK) *M. lepraemurium* was prepared by boiling a suspension of living organisms for 20 min.

Mouse inoculations. Bacteria were injected either into the left or right hind footpad (LHFP or RHFP) in a volume of 0.04 ml or i.v. in a volume of 0.2 ml. Lymphoid cells were injected i.v.

Lymphoid cells. Spleens, popliteal lymph nodes (PLN), or mesenteric lymph nodes (MLN) were re-

moved and dissociated by compression through stainless-steel screens. The cells were washed in Hanks balanced salt solution, total and viable (trypan blue exclusion) leukocyte counts were made, and the cells were adjusted to a density appropriate for injection.

In several experiments, spleen cells were depleted of plastic-adherent cells. The cells were suspended in Hanks balanced salt solution containing 5% fetal calf serum at a density of 10^7 leukocytes/ml. Volumes (50 ml) of cell suspension were placed in disposable polystyrene tissue culture flasks having a surface area of 150 cm^2 (Corning no. 25120). The flasks were incubated at 37°C for 45 min, when the supernatant cells and medium were decanted. The adherent cells were rinsed with Hanks balanced salt solution, and this supernatant was added to the first one. The nonadherent cells were concentrated by centrifugation (500 × *g*) and counted. The proportion of adherent cells was estimated by comparing the preincubation total cell count with the count of nonadherent cells. In some experiments the spleens were weighed before the cells were dissociated.

Footpad measurements. The thicknesses of the left and right hind feet were measured with dial gauge calipers. Since mice were usually immunized in the LHFP, footpad swelling was expressed as the arithmetic mean difference in thickness of the hind feet (left minus right) in millimeters, based on a group size of 10 mice.

Adoptive immunity. Recipient mice were sublethally irradiated by total body exposure to 500 rads from a ^{137}Cs source. They were then challenged i.v, with 10^5 viable R1Rv organisms, after which lymphoid cells were injected i.v. (10). Two types of control mice were used: those that were given no cells and those given spleen cells from normal unimmunized donors. Fourteen days later the mice were killed, the spleens were homogenized, and viable counts of R1Rv organisms per spleen were made. The geometric mean viable count from each group of five cell recipient mice was subtracted from the geometric mean count obtained from controls that had received no cells. These differences, when positive, were a measure of adoptive immunity in $\log_{10}$ units and were evaluated statistically. Resistance of actively immunized mice to BCG or R1Rv organisms was evaluated in the same way (Fig. 1 and 2).

Bacterial counts. Spleens and livers were homogenized with Potter-Elvehjem homogenizers, and feet were processed in a VirTis homogenizer. Viable BCG and R1Rv organisms were enumerated by quantitative culture on Middlebrook 7H-10 agar plates. The sensitivity of the method was such that as few as 50 bacilli (1.7 $\log_{10}$ units) per spleen or footpad, but no less than 1,000 bacilli per liver, could be detected. Suspensions containing living or HK *M. lepraemurium* were appropriately diluted in sterile saline; then quantitative smears were made on Reich slides, stained with phenol auramine, and counted using a fluorescence microscope (14).

Statistical analysis. Viable and total bacterial counts were expressed in $\log_{10}$ units and evaluated by analysis of variance. Group means were compared by the Dunnett or Q test as deemed appropriate (2, 22).

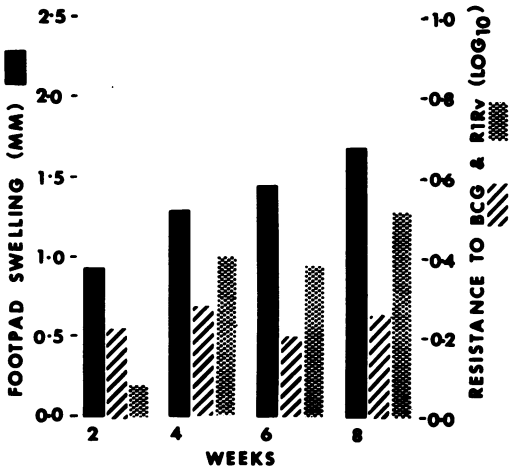

FIG. 1. *Resistance of mice to i.v. challenge with either M. bovis BCG or M. tuberculosis R1Rv organisms after footpad infection with M. lepraemurium. Solid bars, footpad swelling at the site of M. lepraemurium infection; diagonal bars, resistance to BCG; stippled bars, resistance to R1Rv.*

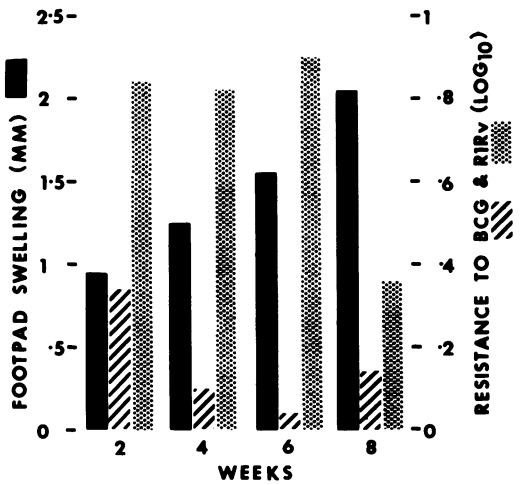

FIG. 2. *Resistance of mice to RHFP challenge with either BCG or R1Rv organisms after LHFP infection with M. lepraemurium. Symbols as in Fig. 1.*

Unless otherwise stated, there were five mice in each experimental group.

RESULTS

Resistance of *M. lepraemurium*-infected mice to BCG and R1Rv organisms. In the first experiment, mice were infected with 10^8 *M. lepraemurium* in the LHFP and, together with normal controls, were later challenged with either 10^5 BCG or 10^5 R1Rv organisms i.v. At the time of challenge the swelling of the LHFP

was measured to establish that the mice were responding to *M. lepraemurium* infection (15). Viable counts of BCG and R1Rv organisms were made from the liver and spleens 14 days after challenge and were expressed relative to the counts obtained from normal control mice. The liver and spleen results were closely similar, and only the latter are presented (Fig. 1).

The *M. lepraemurium*-infected mice developed swelling of the LHFP and concomitant resistance to BCG. The levels of such resistance were consistent, ranging from 0.27 to 0.36 $\log_{10}$ units, with a mean value of 0.30. The resistance observed at a particular time point was sometimes statistically significant ($P < 0.05$) and sometimes not, but analysis of the pooled data yielded a highly significant ($P < 0.01$) value. Resistance to R1Rv organisms did not appear until week 4, but was greater, by approximately 0.20 $\log_{10}$ units, than that expressed against BCG. At weeks 4, 6, and 8 the resistance against R1Rv organisms was highly significant ($P < 0.01$).

It was thought that the low resistance to BCG and R1Rv organisms in the spleens of *M. lepraemurium*-infected mice might be due to enrichment of that organ with respect to suppressor cells (23). In that case, greater resistance to BCG and R1Rv might be expressed at a peripheral site. Mice were therefore infected with 10^8 *M. lepraemurium* in the LHFP. At 2-week intervals for 8 weeks, swelling of that foot was measured and then the RHFP was challenged with either 10^4 BCG or 10^4 R1Rv organisms. Viable counts of mycobacteria in the RHFP were made 14 days after challenge and compared with those obtained from control mice (Fig. 2). The swelling of the LHFP confirmed that the mice had developed a cell-mediated immune response to *M. lepraemurium*. Resistance to BCG ($P < 0.01$) was evident only at week 2. Impressive resistance to R1Rv was observed at 2, 4, and 6 weeks, and a much lower level was observed at 8 weeks. The last result probably represents experimental variation rather than a real diminution of immunity.

Because these two experiments revealed that the antimycobacterial immunity induced by *M. lepraemurium* was better detected by use of R1Rv rather than BCG as the target strain, the former was used in all subsequent experiments.

Transfer of adoptive antituberculosis immunity with lymphoid cells from mice immunized with either BCG or *M. lepraemurium*. Donor mice were immunized with either 10^8 live *M. lepraemurium* or 10^6 BCG organisms in the LHFP. Six weeks later, left PLN, MLN, and spleen cells were harvested from 30, 12, and 6 mice, respectively, from each group of donors. Lymphoid cells (10^8) from each source were injected i.v. into separate groups of five irradiated recipients after challenge with R1Rv organisms. The adoptive immunity conferred by these cell populations is shown in Fig. 3.

In conformity with earlier experiments, cells obtained from the left PLN and spleens of BCG-immunized mice conveyed much higher levels of adoptive immunity than MLN cells (17). PLN, MLN, and spleen cells from *M. lepraemurium*-infected mice conferred closely similar levels of adoptive immunity, of a lesser but still highly significant ($P < 0.01$) magnitude.

Transfer of spleen cells from mice immunized with HK *M. lepraemurium* i.v. In earlier studies, it was shown that a large i.v. dose of live or HK *M. lepraemurium* suppressed the cell-mediated immunity induced by live bacilli inoculated in the footpad (11). It was therefore thought that spleen cells from mice immunized i.v. with living or dead *M. lepraemurium* not

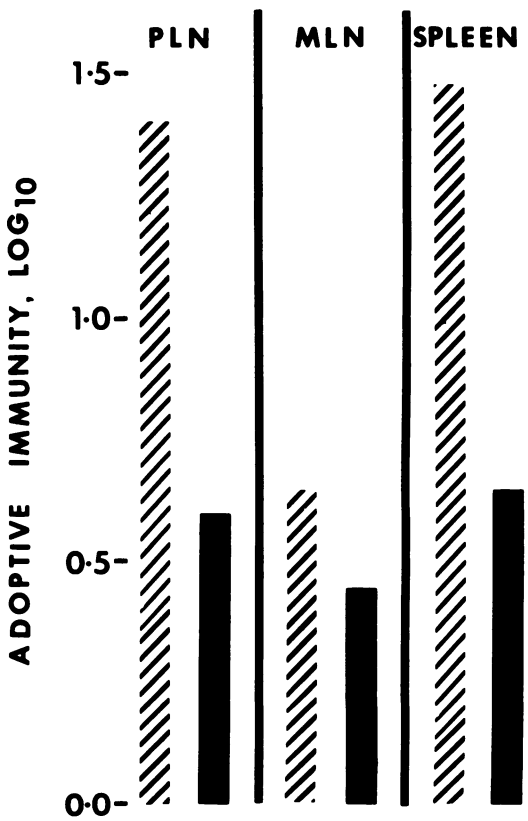

FIG. 3. *Transfer of adoptive immunity to M. tuberculosis R1Rv with left PLN, MLN, and spleen cells from mice immunized in the LHFP with either BCG (diagonal bars) or M. lepraemurium (solid bars).*

only would be unable to confer adoptive antituberculosis immunity, but also might suppress that function in lymphocytes obtained from mice inoculated in the LHFP with live BCG or live *M. lepraemurium*. The subsequent experiments were designed to explore this hypothesis.

In the first experiment, mice were immunized with *M. lepraemurium* as follows: 10^9 HK organisms i.v., 10^7 live organisms i.v., or 10^8 live organisms in the LHFP. Four and ten weeks later, spleens were removed from these groups of mice and unimmunized controls and weighed. The spleen cells were then dissociated and counted, and the plastic-adherent cells were removed. The nonadherent cells were counted and then injected i.v. into irradiated recipients that had been challenged with R1Rv. The dose of injected cells per recipient mouse was that number of nonadherent cells derived from a single donor spleen (one donor equivalent). Viable counts were made from the spleens 14 days later.

Table 1 shows the spleen weights and cell content. The spleen weight was not increased above normal values in mice infected in the LHFP. Mice infected with live bacilli i.v. developed slowly progressive splenomegaly, whereas mice receiving HK organisms i.v. rapidly developed splenomegaly, which later subsided. The number of leukocytes per spleen varied with the spleen weight, but the relationship was not direct. The proportion of adherent cells per spleen was closely similar in all groups of mice at both time points. Consequently, recipients of cells from donors with splenomegaly received more cells than the other recipients.

R1Rv organisms multiplied to a lesser, but statistically insignificant, extent in recipients of normal spleen cells than in controls that received no cells (Fig. 4). Consequently, in evaluating the effect of spleen cell transfers, the recipients of cells from immunized donors were compared with recipients of normal cells. This procedure was also followed in other experiments of similar design (Figs. 5 and 9). At 4 weeks (Fig. 4), significant adoptive immunity was conferred by spleen cells from all the groups of immunized mice ($P < 0.01$), but cells from LHFP-immunized mice were more protective ($P < 0.05$) than those from mice immunized with HK bacilli i.v., even though recipients of the latter received many more cells (Table 1). Cells obtained at 10 weeks from mice immunized with either live organisms in the LHFP or HK bacilli i.v. also conferred substantial adoptive immunity ($P < 0.01$) but cells from mice infected with live *M. lepraemurium* i.v. did not confer significant protection, despite the large number of cells transferred (Table 1).

In the preceding experiment, the comparative immunizing ability of the spleen cells from various types of donors was impossible to evaluate because differing numbers of spleen cells were transferred. To obtain more comparable data, an experiment was designed in which equal numbers of lymphoid cells were injected into recipients.

Mice were immunized with either 10^8 *M. lepraemurium* in the LHFP, 10^8 *M. lepraemurium* i.v., or 10^9 HK *M. lepraemurium* i.v. Normal mice were set aside as prospective spleen cell donors. Four weeks later, spleen cells were obtained from all these groups of mice and either 10^8 or 5×10^7 nonadherent spleen cells were injected into separate groups of five R1Rv-challenged, irradiated recipient mice. Viable counts were obtained from their spleens 14 days later.

The viable counts obtained from recipients of 10^8 and 5×10^7 cells from the same source were closely similar. Accordingly, these groups were pooled, providing 4 groups of 10 mice for data analysis. The viable counts obtained from recipients of sensitized cells were compared with the counts obtained from recipients of normal cells that represent zero immunity in Fig. 5. Although

TABLE 1. *Effect of immunization with M. lepraemurium preparations upon the spleen*

Week	Immunization with *M. lepraemurium*	Spleen wt (mg)	Spleen leukocytes		Counts of acid-fast bacilli ($\log_{10}$)	
			Total (10^8)	Nonadherent (%)	LHFP	Spleen
4	None	102	1.1	52	ND[a]	ND
	10^8 in LHFP (live)	101	1.5	50	8.49	5.55
	10^7 i.v. (live)	224	3.2	59	ND	7.29
	10^9 i.v. (HK)	422	5.3	58	ND	7.43
10	None	93	1.3	58	ND	ND
	10^8 in LHFP (live)	115	1.7	47	8.80	5.82
	10^7 i.v. (live)	635	4.5	52	ND	8.43
	10^9 i.v. (HK)	237	2.5	58	ND	7.18

[a] ND, Not done.

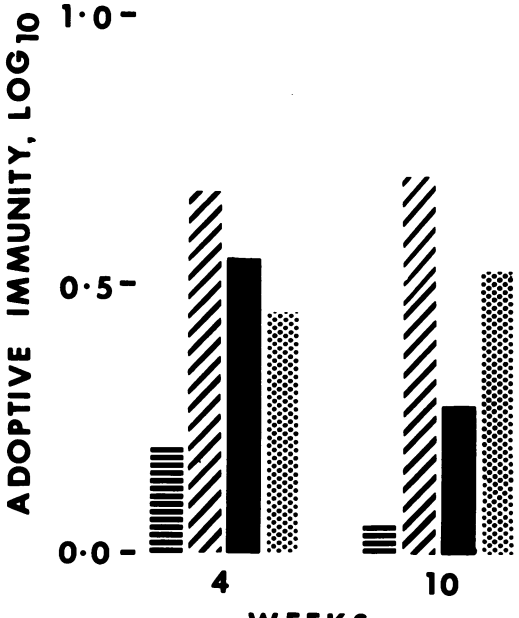

FIG. 4. *Adoptive immunity to M. tuberculosis R1Rv after transfer of spleen cells obtained 4 and 10 weeks after inoculation with M. lepraemurium as follows: 10^8 live bacilli in the LHFP (diagonal bars); 10^7 live bacilli i.v. (solid bars); or 10^9 HK bacilli i.v. (stippled bars). The horizontal broken bars denote recipients of spleen cells from normal mice.*

the levels of adoptive immunity were small, all were highly significant ($P < 0.01$). The differences between the various groups of recipients of sensitized spleen cells were not statistically significant, but the data closely resemble those in Fig. 4 for week 4 and suggest that the least protective cells were from mice immunized with HK *M. lepraemurium* i.v.

Induction of specific immunity by BCG in mice preimmunized with *M. lepraemurium*. Groups of mice were immunized with either 10^7 live or 10^9 HK *M. lepraemurium* i.v. Four weeks later, these mice and a group of normal mice were infected with 10^6 BCG organisms in the LHFP. Six weeks after BCG immunization (10 weeks after preimmunization with *M. lepraemurium*), these mice were killed and used to determine spleen weight, viable BCG in the liver and spleen, total counts of mycobacteria in spleen homogenates, and transfer of antituberculosis immunity with spleen cells, using one donor equivalent of nonadherent spleen cells per recipient.

The mean spleen weight was 92 mg for normal mice. The results for mice inoculated i.v. with either *M. lepraemurium* or HK *M. lepraemurium* were 605 and 284 mg, respectively. The corresponding yields of unseparated cells per spleen were 1.4×10^8, 6.5×10^8, and 3.4×10^8. The spleens of mice immunized with live and HK bacilli i.v. contained 8.64 and 7.29 $\log_{10}$ stainable bacilli, respectively, at 10 weeks. At that time no viable BCG ($<10^3$) were detected in the livers of any group of donors, but there were 2.57 $\log_{10}$ viable BCG organisms in the spleens of mice that had not been preimmunized with *M. lepraemurium* preparations. Even fewer viable BCG (<1.70 $\log_{10}$) were detected in the spleens of mice that had been preimmunized with *M. lepraemurium* preparations, indicating that these mice expressed antibacterial immunity against BCG. Groups of recipients received different numbers of nonadherent spleen cells (Fig. 6B), reflecting differences in donor spleen size. All recipients expressed higher levels of adoptive immunity than mice immunized solely with *M. lepraemurium* preparations (Fig. 6A). However, cells from mice preimmunized with live *M. lepraemurium* organisms conveyed significantly lower immunity ($P < 0.01$) than those from mice that were not preimmunized, suggesting that BCG vaccination was less effective in the former animals.

Transfer of adoptive immunity with spleen cells of suppressed and unsuppressed mice. Three groups of donor mice were immunized with 10^8 live *M. lepraemurium* in the LHFP. One group of mice received no further

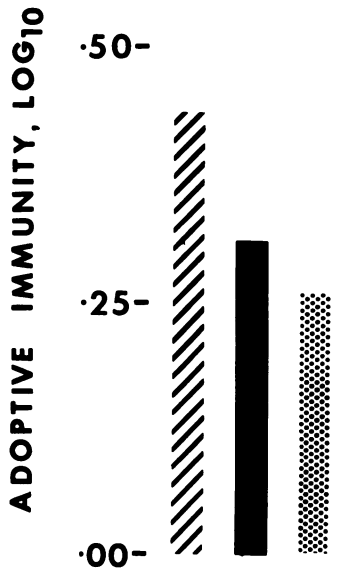

FIG. 5. *Adoptive immunity to R1Rv in recipients of spleen cells from donors that had been immunized with M. lepraemurium as follows: 10^8 live bacilli in the LHFP (diagonal bar) or i.v. (solid bar), or 10^9 HK bacilli i.v. (stippled bar).*

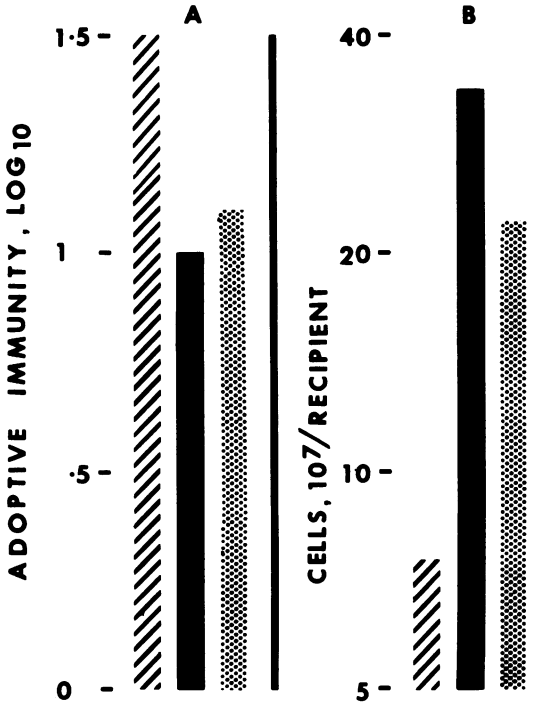

FIG. 6. *Transfer of adoptive immunity to R1Rv with spleen cells from donors that either were vaccinated with 10^6 live BCG in the LHFP alone (diagonal bars) or had been preimmunized with 10^7 live (solid bars) or 10^9 HK (stippled bars) M. lepraemurium i.v. (A) Adoptive immunity. (B) Number of nonadherent spleen cells (one donor spleen equivalent) injected into individual recipient mice.*

treatment, but the remaining groups were given concomitant i.v. injections of either 10^7 live or 10^9 HK *M. lepraemurium* bacilli. Footpad measurements were made for 12 weeks; at 6 and 12 weeks, counts of acid-fast bacilli were made from the LHFP, liver, and spleen. The spleens were also weighed, and the ability of their cells to convey adoptive immunity was determined.

The footpad measurements (Fig. 7) showed that the early footpad swelling that signifies the induction of cell-mediated immunity was suppressed in mice given *M. lepraemurium* i.v. Such suppression was associated with splenomegaly, similar to that shown in Table 1. There was closely similar growth of *M. lepraemurium* organisms in the LHFP of the three groups of infected mice, and there was little dissemination of organisms from the feet of mice inoculated only in the LHFP (Fig. 8). There was substantial proliferation of mycobacteria in the livers and spleens of mice given live *M. lepraemurium* bacilli i.v., and almost 10^{10} organisms per mouse were present at 12 weeks. There was little difference between the numbers of HK bacilli found in the livers and spleens at 6 and 12 weeks. Analysis of the adoptive immunity data (Fig. 9) revealed that at both 6 and 12 weeks the cells from immunized mice conveyed significantly greater protection ($P < 0.05$ to $P < 0.01$) than normal cells, recipients of which were not significantly more immune than mice that received no cells. It is notable that the spleen cells of i.v.-infected mice obtained at week 12 conveyed protective immunity even though these animals had extensive disseminated infection and carried a heavy burden of *M. lepraemurium* organisms in their livers and spleens.

Suppression of BCG-sensitized cells by spleen cells from *M. lepraemurium*-immunized mice. One group of donor mice was immunized with 10^6 BCG in the LHFP. Two weeks later, another group of donors received 10^7 live *M. lepraemurium* organisms i.v., and a third group was given 10^9 HK *M. lepraemurium* i.v. Six weeks after BCG immunization (4 weeks after immunization with *M. lepraemurium*), spleen cells were prepared from each group of donors and four groups of irradiated recipients were challenged with R1Rv i.v. Group 1 received no cells, and group 2 received one donor equivalent of cells from BCG-vaccinated mice only. The remaining mice received one donor equivalent of BCG-sensitized cells and also one donor equivalent of cells from mice immunized with either live (group 3) or HK (group 4) *M. lepraemurium* i.v. Viable counts of R1Rv per spleen were obtained 14 days later (Fig. 10). A high level of protection was expressed in all recipients

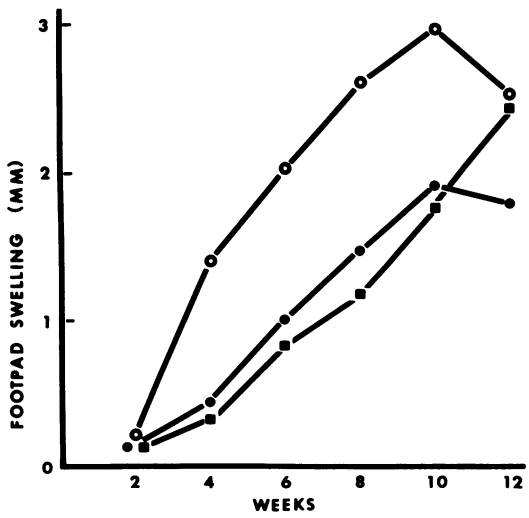

FIG. 7. *Swelling of the LHFP resulting from the inoculation of 10^8 live M. lepraemurium in the LHFP, either alone (○) or accompanied by 10^7 live (●) or 10^9 HK (■) M. lepraemurium i.v.*

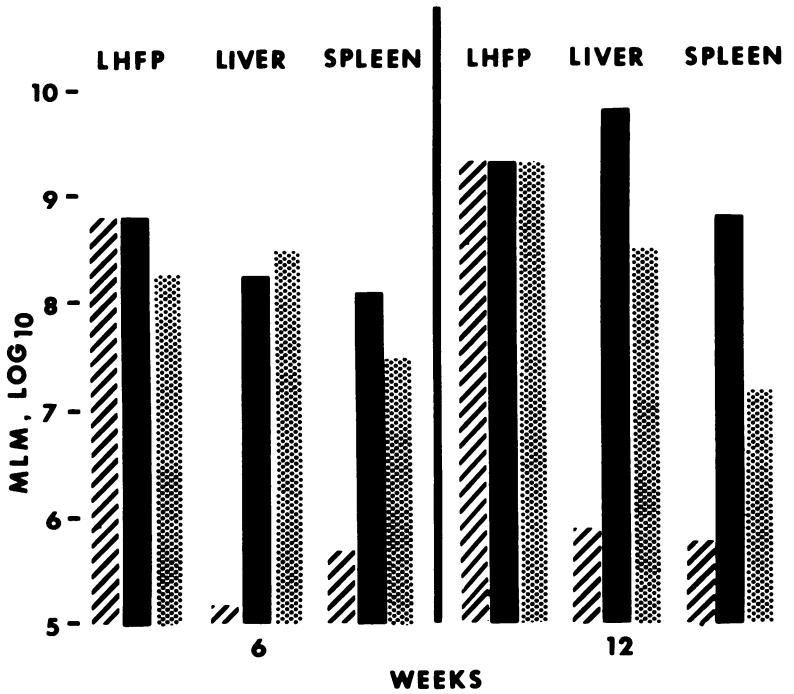

FIG. 8. *Acid-fast bacilli in the LHFP, liver, and spleen after inoculation of 10^8 live M. lepraemurium in the LHFP, either alone (diagonal bars) or accompanied by 10^7 live (solid bars) or 10^9 HK (stippled bars) M. lepraemurium i.v.*

of BCG-sensitized cells, but recipients of cells from HK *M. lepraemurium*-immunized mice were significantly less protected ($P < 0.01$) than mice that received BCG-sensitized cells only.

Transfer of antigen with spleen cells. The removal of plastic-adherent spleen cells had a dual purpose: (i) to remove the excessive number of macrophages in the spleens of mice that had received live or HK *M. lepraemurium* i.v. and (ii) to minimize the transfer of *M. lepraemurium* bacilli that resided in donor spleen macrophages. In the entire series of experiments, between 40 and 60% of spleen cells were found to adhere to plastic (Table 1). The proportion of adherent cells was influenced neither by the treatment received by the donor nor by the spleen weight. Counts of *M. lepraemurium* were made on non-adherent spleen cell samples and whole spleen homogenates from the same groups of animals. The results of a typical experiment (Table 2) show that a substantial proportion of mycobacteria was removed by allowing the spleen cells to adhere to plastic. Even so, in the case of spleens that were heavily infected, the removal of 60% of the organisms still permitted the transfer of large numbers of bacilli into recipient mice. No attempt was made to determine whether the transferred bacilli were intra- or extracellular.

Transfer of adoptive immunity with graded numbers of spleen cells from *M.*

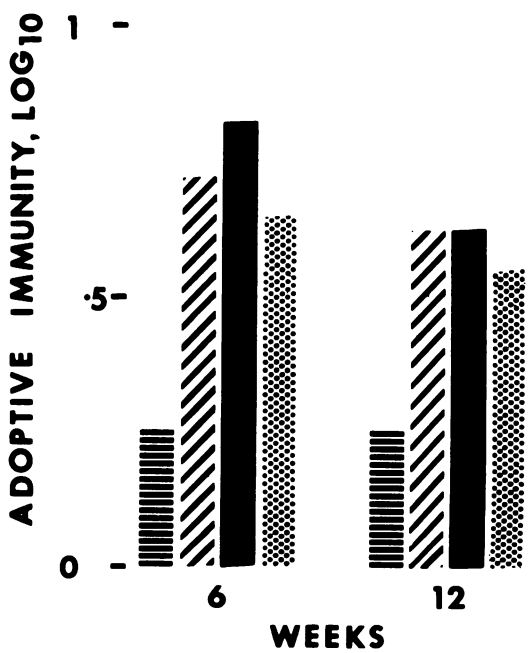

FIG. 9. *Adoptive immunity to M. tuberculosis R1Rv in recipients of spleen cells from normal mice (horizontal bars) and mice inoculated with 10^8 live M. lepraemurium in the LHFP, either alone (diagonal bars) or concurrent with 10^7 live (solid bars) or 10^9 HK (stippled bars) M. lepraemurium i.v.*

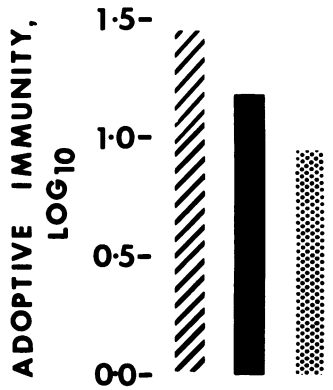

FIG. 10. *Suppression of BCG-induced adoptive antituberculosis immunity by addition of spleen cells from M. lepraemurium-immunized mice. Recipient mice received either BCG-sensitized spleen cells only (diagonal bar) or mixtures of BCG-sensitized cells and cells from mice immunized with 10^7 live (solid bar) or 10^9 HK (stippled bar) M. lepraemurium i.v.*

TABLE 2. *Removal of M. lepraemurium by adherence of spleen cells to plastic*

Immunization[a] with *M. lepraemurium*	Acid-fast bacilli/spleen equivalent		Reduction of acid-fast bacilli (%)
	Unseparated	Nondherent	
10^8 in LHFP (live)	6.2×10^5	8.8×10^4	86
10^7 i.v. (live)	1.7×10^8	7.0×10^7	60
10^9 i.v. (HK)	1.8×10^6	3.1×10^5	83

[a] Mice were immunized 10 weeks before spleens were removed.

***lepraemurium*-infected and BCG-infected donors.** Mice were immunized with either 10^6 live BCG or 10^8 live *M. lepraemurium* organisms in the LHFP. Six weeks later, spleen cells from these mice and normal controls were obtained, and 3×10^6 to 100×10^6 cells were injected i.v. into irradiated recipients that had been challenged i.v. with R1Rv organisms. Viable counts of R1Rv organisms were obtained from the spleens of mice 14 days later, and the results were expressed relative to the counts obtained from control mice that received no cells (Fig. 11). Transfer of 25×10^6 to 100×10^6 normal spleen cells to irradiated recipients inhibited the growth of R1Rv organisms to a trivial degree, in a dose-related fashion. Cells from BCG-infected mice conferred a high level of adoptive immunity: as few as 3×10^6 cells produced over 1 $\log_{10}$ unit of protection. The dose-response relationship was rather flat, a 30-fold dilution of sensitized cells resulting in only a 2-fold (0.30 $\log_{10}$ units) reduction in immunity.

Cells from *M. lepramurium*-infected mice conferred significantly greater immunity than normal cells ($P < 0.01$), but much less immunity than BCG-sensitized cells ($P < 0.01$). Although the *M. lepraemurium*-sensitized cells were titrated only over the limited range of 25×10^6 to 100×10^6 cells, the dose response was again flat and approximately parallel to that obtained with BCG-sensitized cells. It is notable that 25×10^6 to 100×10^6 *M. lepraemurium*-sensitized cells conferred as much resistance to R1Rv as that observed in actively immunized mice that were challenged i.v. (Fig. 1).

DISCUSSION

This study establishes that reciprocal cross-reactive immunity exists between the *M. tuberculosis* species complex (which includes *M. bovis* BCG) and *M. lepraemurium*. In an earlier study (14), it was shown that immunization with BCG inhibits the proliferation of *M. lepraemurium* at a subcutaneous site. In this paper, we have shown that the proliferation of BCG and *M. tuberculosis* R1Rv is inhibited in *M. lepraemurium*-infected mice. Such inhibition was more easily demonstrable against R1Rv, but this should not be construed to mean that R1Rv is more closely related, antigenically, to *M. lepraemurium* than BCG. A more probable explanation is that whereas the growth of BCG and R1Rv is inhibited to the same extent in immunized hosts, R1Rv proliferates more rapidly in normal hosts and so appears to be more suscep-

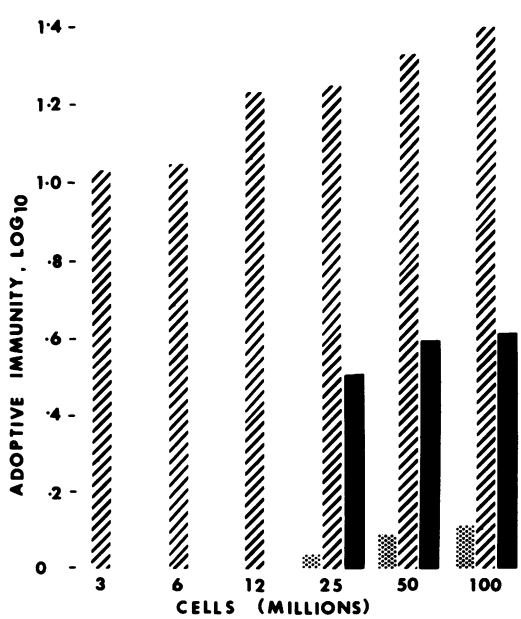

FIG. 11. *Adoptive immunity to M. tuberculosis R1Rv in recipients of spleen cells from normal (stippled bars), BCG-immunized (diagonal bars), or M. lepraemurium-infected (solid bars) mice.*

tible to the immune effector mechanism.

In view of the above results, it was anticipated that lymphoid cells from mice infected subcutaneously with *M. lepraemurium* would adoptively immunize mice against *M. tuberculosis*. This proved to be the case, but the degree of protection was much lower than in the homologous system, in which mice were immunized with BCG. A similar low level of cross-reactive antituberculosis immunity is generated in mice immunized with irradiated *M. leprae* (18, 19).

There are two technical considerations that might have influenced the assay of adoptive immunity using spleen cells from i.v.-immunized mice. It was known that i.v. immunization with high doses of mycobacteria produces splenomegaly, a substantial but undetermined part of which comprises macrophages within granulomas (11). It was thought that transfer of excessive numbers of such macrophages was undesirable for three reasons. Some spleen macrophages would contain living or dead *M. lepraemurium* organisms, or both, and so constitute the source of transferred antigens; macrophages might exert a nonspecific suppressive function in the adoptive host (5, 25); and, even if functionally inert, the macrophages would dilute the spleen lymphocytes, so that, of a given number of transferred spleen cells, a lower proportion would comprise specifically sensitized mediator cells. To circumvent these problems, two corrective procedures were used. The spleen cells were incubated over a polystyrene surface at 37°C to remove adherent cells, including macrophages; and the cells were transferred as donor spleen equivalents rather than absolute numbers of leukocytes to ensure that different groups of recipients received adequate numbers of lymphocytes. In practice, spleen cell transfers yielded similar results, regardless of whether the cells were given as donor equivalents or absolute cell numbers (compare Fig. 5 and 6). The removal of adherent cells achieved very little in that the proportion of cells removed was similar for normal spleen cells, which contained relatively few macrophages, and enlarged spleens, which contained many. This observation may be attributable to the fact that cells other than macrophages may adhere to polystyrene (16). Moreover, removal of adherent cells did not prevent the transfer of substantial numbers of *M. lepraemurium* bacilli.

The influence of the transfer of *M. lepraemurium* organisms from donors to recipients deserves some consideration. Two mutually exclusive effects appear to be possible. First, an excessive burden of *M. lepraemurium* bacilli might suppress the donor effector lymphocytes and block their expression. Alternatively, the *M. lepraemurium* bacilli might induce a primary immune response in the recipients, independent of the transferred spleen cells. The first alternative might explain the suppressive effects of spleen cells from mice inoculated i.v. with large doses of *M. lepraemurium* bacilli. The second alternative implies that true adoptive immunity was not conferred by cells from *M. lepraemurium*-infected mice, but rather that the recipients generated a primary immune response to *M. lepraemurium*. This possibility cannot be excluded categorically, but it fails to explain the transfer of higher levels of antituberculosis immunity with spleen cells from footpad-infected mice, whose spleens contained few bacilli.

We were surprised that mice inoculated i.v. with large doses of *M. lepraemurium*, live or HK, generated spleen cells that conveyed adoptive immunity to *M. tuberculosis*, because mice so immunized suppress the induction of cell-mediated immunity that follows footpad infection with *M. lepraemurium* (11). However, the protective immunity conferred by i.v.-immunized, putatively suppressed mice was often, if not significantly, lower than that conferred by footpad-infected, unsuppressed mice. This was particularly evident in the case of spleen cells obtained from mice 10 weeks after i.v. infection with 10^7 live *M. lepraemurium*. At that time the bacillary load of *M. lepraemurium* organisms would be approximately 10^9, and those mice were expected to die within 4 to 6 weeks.

A peculiar situation prevailed in mice with *M. lepraemurium* footpad infection and concomitant i.v. challenge with live homologous organisms. The spleen cells of such mice conveyed good antituberculosis immunity as late as 12 weeks after i.v. infection. This result suggests that the cell-mediated immunity engendered by the footpad infection in some way helped to maintain protective cells in the spleen (Fig. 10), whereas mice infected only i.v. lacked such cells (Fig. 5). It appears that the suppressive action of i.v. infection may have been offset by the effector mechanism induced by footpad infection. The influence of i.v. challenge with *M. lepraemurium* upon BCG-induced protective immunity was adverse in that the level of adoptive immunity was reduced in recipients of cells from mice immunized with both BCG and *M. lepraemurium*; also, addition of *M. lepraemurium*-sensitized cells to BCG-sensitized cells depressed the activity of the latter.

Throughout the entire study, the measured differences in the levels of adoptive immunity conferred by cells from putatively unsuppressed and suppressed donors were disappointingly

small, even when statistically highly significant. Several explanations of this are offered. The dose-response curve at high doses of cells was very flat (Fig. 11). Consequently, at the commonly used dose of 10^8 cells, suppression or elimination of 75% of the effector lymphocytes would have no measurable effect on the transfer of adoptive immunity. In retrospect, more impressive results might have been obtained if much smaller numbers of lymphocytes had been used for the adoptive immunity experiments. A second possibility is that the antigenic determinants shared by *M. lepraemurium* and the *M. tuberculosis* complex govern T-cell-mediated protective immunity but do not induce suppressor cells. Such a phenomenon has been described in the humoral immune response to lysozyme in which the antigenic determinants that induce helper and suppressor T cells, respectively, are different (1). Should a similar situation prevail in our system, it would account for some paradoxical observations: cross-reactive protective functions were substantial, whereas cross-reactive suppressive functions were often trivial; and cells that conferred protective immunity were present in the spleens of putatively suppressed mice (5) that were dying from *M. lepraemurium* infection. Another possibility is that suppressor T cells have a short functional life-span, analogous to the short-lived cells that mediate tuberculin delayed-type hypersensitivity (12), in contrast to the long-lived effector T cells that mediate antituberculosis immunity (13). Since the assay of antimycobacterial adoptive immunity takes 14 days, the action of short-lived cells might go undetected. Alternatively, the effective expression of suppressor cell function may depend on cooperation between different subclasses of suppressor cells (3) or critical juxtapositions with other cells. Such conditions may prevail in the spleens of donor mice but may not survive spleen cell dissociation. Finally, humoral suppressors may be important. These may include infection-enhancing antimycobacterial antibody (8), circulating antigens (4), immune complexes (9), or anti-idiotypic antibodies (24). Such factors were not investigated in this current study.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grants AI-07015 and AI-17306 from the National Institutes of Health. We express our thanks to Roberta Morgan for excellent technical assistance. Much of this work was performed at Trudeau Institute Inc., Saranac Lake, N.Y.

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