INFECTION AND IMMUNITY, Mar. 1988, p. 660–664
0019-9567/88/030660-05$02.00/0

Vol. 56, No. 3

Recombinant Interleukin-2 Limits the Replication of *Mycobacterium lepraemurium* and *Mycobacterium bovis* BCG in Mice

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Received 2 September 1987/Accepted 24 November 1987

BALB/c mice were infected with *Mycobacterium lepraemurium* in the footpad or with *Mycobacterium bovis* BCG intravenously with 5×10^7 bacilli. Recombinant interleukin-2 (IL-2) was injected intraperitoneally as a single dose (20,000 U), as a single course of five injections (400 U each), or as a 6-month course starting 3 days after the *M. lepraemurium* infection. BCG-infected mice received a single dose (1,000 U) or five daily injections of 100 or 1,000 U each. IL-2 significantly reduced the total bacterial counts in the footpad, lymph nodes, and liver of *M. lepraemurium*-infected mice (50 to 85%) by 6 months and viable counts in the spleen (30 to 50%) by 60 days after BCG infection. The courses of IL-2 started at 60 days were more effective than those started at 3 days after *M. lepraemurium* infection ($P < 0.05$ to 0.001), and for BCG, 100 U of IL-2 was better than 1,000 U ($P < 0.05$ to 0.01). These results indicate that IL-2 limits mycobacterial infections in mice and raise the question of its possible use in humans.

Mycobacteria are a major health problem, and drug resistance, especially of nontuberculous mycobacteria, is of real concern. For this reason, study of novel modes of therapy is important, especially as the acquired immunodeficiency syndrome epidemic has increased the cases of nontuberculous mycobacterial infections. Interleukin-2 (IL-2) may have an important role in mycobacterial infection since it is known that IL-2 induces the production of gamma interferon and other lymphokines (9), which in turn activate murine macrophages and human monocytes to inhibit the growth of a variety of mycobacteria (15, 19; R. C. Bucklin and A. J. Crowle, J. Leukocyte Biol. 40:324–325, 1986). There is also evidence to show that IL-2 affects the response of lymphocytes to mycobacterial antigens as it reverses the T-cell unresponsiveness in some human and experimental systems (2, 4, 10, 17; but see reference 1). However, there is no report on whether in vivo administration of IL-2 limits the replication of mycobacteria. Against this background, we studied the effect of IL-2 on the ability of mice to handle *Mycobacterium lepraemurium* and *Mycobacterium bovis* BCG infection and showed that therapy reduces the total as well as the viable bacterial counts.

MATERIALS AND METHODS

Mice. Female BALB/c mice bred locally were 8 to 12 weeks old at the beginning of each experiment.

Source of organisms for infection. *M. lepraemurium* (Douglas strain) was grown in Parkes strain mice (3) and isolated from the liver and spleen by the second method of Draper (8). *M. bovis* BCG was the Glaxo strain and was stored in suspension at −70°C.

IL-2. Human recombinant IL-2 (7) was kindly supplied by Biogen. IL-2 was injected as a single shot, as a single course of five injections, or as multiple monthly courses.

Infection of mice. (i) ***M. lepraemurium.*** Mice were placed randomly in groups and then injected in the hind footpad with *M. lepraemurium* at a dose of 5×10^7 bacilli. They were randomized again, and 0.2 ml of IL-2 in 1% fetal calf serum was injected intraperitoneally. The various IL-2 treatments were as follows: (i) five injections of 400 U of IL-2 given at 3-day intervals starting on day 3 of infection, (ii) similar IL-2 treatment but repeated courses every month for 6 months, (iii) similar repeated courses every month for 4 months starting on day 60 of infection, and (iv) a single injection of 20,000 U of IL-2 on day 3 of infection. The control mice received fetal calf serum in saline. The course of *M. lepraemurium* infection was followed by assessing the number of bacteria in various tissues. Since *M. lepraemurium* cannot be easily grown in vitro, viable counts were not undertaken. The total bacterial counts in the footpad, lymph nodes, and liver after *M. lepraemurium* infection were assessed at monthly intervals after the concentration of acid-fast bacilli in the tissues (6). Briefly, the tissues were homogenized in 4% NaOH containing 0.2% potassium alum, incubated at 37°C for 25 min, washed twice in phosphate buffer (0.067 M, pH 6.7), and suspended in 0.1% albumin saline. A volume of 5 ml was used for the liver and footpads and 1 ml for the lymph nodes. A measured volume was then transferred to multitest slides (Flow Laboratories, Inc., McLean, Va.), stained by the Ziehl-Neelsen method, and counted (16). No clumping of bacteria was seen. The inflammation at the site of injection of the bacteria was assessed by measuring the footpad thickness in groups of eight mice with a dial caliper and correcting for the thickness of the uninfected footpad.

(ii) **BCG.** Four groups of randomized mice were infected intravenously with 5×10^7 BCG cells, and on day 3 of infection, one group received fetal calf serum in saline. The remaining three groups of mice were injected intraperitoneally with various regimens of IL-2 as follows: (i) five daily injections of 100 U (two experiments), (ii) five daily injections of 1,000 U, or (iii) a single injection of 1,000 U. The total and viable bacterial counts in the spleen were done at regular intervals. The spleens were first homogenized in 0.1% albumin in distilled water and sonicated briefly for 5 s with a sonicator (MSE U.K.) set at 80% of the maximum. Three $\log_{10}$ dilutions were made, 100 μl was plated in triplicate on 7H11 agar, and the colonies were counted at 21

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days. The CFU counts were based on the highest dilution showing distinct colonies.

For total bacterial counts, the remaining homogenate was mixed with an equal volume of 4% NaOH containing 0.2% potassium alum, treated as for *M. lepraemurium*, and sonicated immediately before smearing. Some clumps of bacteria were seen, and an estimate of the number of bacteria in them was made. All the results are based on groups of five mice assessed individually and are expressed as mean counts per organ.

Statistics. Statistical analysis was based on analysis of variance of log-transformed data (dependent variable), with treatments and time points as the independent factors. Significance was based on the Student's *t* test, using the mean square error and degrees of freedom from the analysis of variance.

RESULTS

Effect of IL-2 on *M. lepraemurium* infection. After the footpad infection, *M. lepraemurium* cells grew progressively at the infection site and spread to the regional lymph node (popliteal) and liver. Two experiments were undertaken to assess the effect of IL-2 on the course of *M. lepraemurium* infection. In the first experiment, a single course of five injections of IL-2 (400 U given every 3 days, starting on day 3 of infection) reduced the *M. lepraemurium* counts in the lymph nodes and liver (Fig. 1). The reduction in the lymph nodes was significant from 3 months onward, and that in liver reached significance at 4 and 6 months as compared with that in the controls. In the footpad, the reduction in the bacterial counts was only 10% that of controls after IL-2 injection, and hence the data are not shown in the figure but appear in Table 2 (experiment 1).

Experiment 2 investigated whether a larger effect could be obtained with IL-2 by repeating the monthly treatments for 6 months or by giving a single large dose on day 3 of infection. It was also important to see whether IL-2 was still effective when the treatment was delayed 60 days, at a time when the infection was already established. The results from these experiments (Table 1) showed that treatment with multiple monthly courses of IL-2 (400 U given five times every 3 days starting on day 3) or a single shot of 20,000 U on day 3 caused a significant reduction in the bacterial counts in the footpad, lymph nodes, and liver. This reduction was seen as early as 1 month in some groups. Even when treatment was delayed for 60 days, monthly courses of IL-2 (400 U given five times every 3 days) were still effective and significant reduction occurred in the liver 1 month later and in the other tissues at 2 months. In fact, the delayed treatment with IL-2 was more effective than comparable treatment started at 3 days. A significant reduction in counts was seen in the footpad and lymph nodes at 4, 5, and 6 months ($P < 0.05$ to 0.001) and in the liver at 4 months ($P < 0.001$). The data from these two experiments are summarized in Table 2. This shows that with the exception of the footpads in experiment 1 (five injections of 400 U each), the mean percent reduction in the bacterial counts at 4 to 6 months in the footpads, lymph nodes, and liver was approximately 60% (range, 51 to 86%).

Effect of IL-2 on BCG infection. It seemed important to determine whether IL-2 affected the viability of mycobacteria or only affected the rate at which dead organisms lost the ability to take up Ziehl-Neelsen stain. For this reason, the effect of IL-2 on BCG infection was studied since viable bacterial counts are readily obtained with this organism. Figure 2 shows the viable bacterial counts in the spleen after BCG infection by the intravenous route. When IL-2 was given as a course of five injections (100 or 1,000 U daily starting on day 3) or as a single shot (1,000 U on day 3), the viable counts were reduced significantly. A single shot of IL-2 was the least effective, while the low-dose course (100 U) of IL-2 appeared more effective than the high-dose course (1,000 U). This paradoxical difference between high- and low-dose treatment was statistically significant at 40, 50, and 60 days ($P < 0.05$ to 0.01). These results are summarized in Table 3. We have no explanation why the higher doses of IL-2 only limited total (i.e., live and dead) bacterial counts in the first 30 days in this experiment. Table 3 also shows a second experiment which confirmed that 100 U of IL-2 lowered the total splenic bacterial counts.

Miscellaneous observation. Infection with *M. lepraemurium* or BCG with or without IL-2 had no effect on the weight of the mice compared with that of a control group of

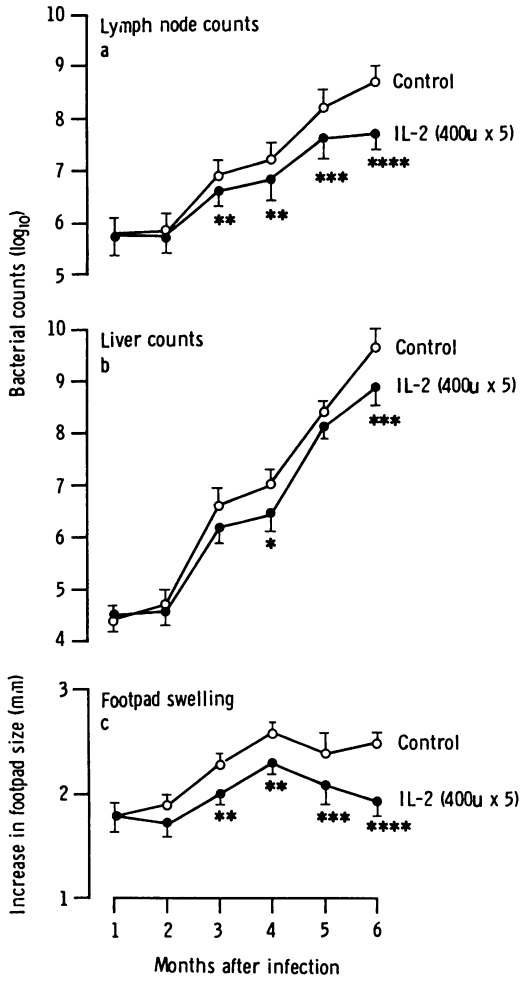

FIG. 1. Effect of IL-2 on bacterial counts and footpad swelling after *M. lepraemurium* infection in BALB/c mice (experiment 1). Mice were infected in the footpad with 5×10^7 *M. lepraemurium* cells. Five injections of 400 U of IL-2 were given at 3-day intervals starting on day 3 after infection. The control mice were infected with *M. lepraemurium* but given saline. The mean counts in the lymph nodes (a) and liver (b) and the swelling in the infected footpad (c) based on groups of five mice are shown with the 95% confidence interval. *, $P < 0.05$; **, $P < 0.02$; ***, $P < 0.01$; ****, $P < 0.001$, compared with controls.

TABLE 1. Effect of various regimens of IL-2 on bacterial counts after *M. lepraemurium* infection in BALB/c mice (experiment 2)[a]

Mo postinfection	Mean bacterial count ± SD (% reduction)			
	Untreated controls	IL-2 treatment		
		Early courses	Late courses	Single shot
Footpad[b]				
1	1.07 ± 0.26	0.94 ± 0.17 (12)		0.41 ± 0.14§ (62)
2	5.40 ± 1.30	3.80 ± 1.80‡ (30)		2.80 ± 1.20† (48)
3	7.60 ± 1.80	5.60 ± 1.10 (26)	4.30 ± 1.1 (43)	4.40 ± 1.40 (42)
4	80.80 ± 19.50	36.30 ± 7.50† (55)	9.40 ± 3.8§ (88)	40.50 ± 8.50† (50)
5	108.90 ± 11.50	30.80 ± 4.40§ (72)	22.00 ± 7.5§ (80)	34.30 ± 6.03§ (69)
6	357.90 ± 82.20	128.00 ± 23.90§ (64)	34.50 ± 5.7§ (90)	115.90 ± 16.00§ (68)
Lymph node[c]				
1	0.05 ± 0.008	0.05 ± 0.008 (0)		0.04 ± 0.009 (26)
2	1.69 ± 0.75	0.95 ± 0.38§ (44)		0.77 ± 0.30§ (54)
3	6.04 ± 1.90	3.90 ± 0.46 (35)	4.10 ± 1.10 (31)	2.20 ± 0.86§ (64)
4	52.60 ± 12.40	30.70 ± 5.20† (42)	16.40 ± 1.80§ (69)	22.70 ± 8.10† (57)
5	107.60 ± 41.10	40.90 ± 18.50* (62)	23.50 ± 7.50§ (78)	49.30 ± 5.10† (54)
6	245.80 ± 65.70	112.70 ± 13.50† (54)	66.10 ± 13.70§ (73)	63.40 ± 10.60§ (74)
Liver[c]				
1	0.007 ± 0.003	0.005 ± 0.0009 (31)		0.003 ± 0.001† (65)
2	0.09 ± 0.02	0.06 ± 0.02 (33)		0.07 ± 0.03 (23)
3	0.58 ± 0.26	0.30 ± 0.06‡ (48)	0.23 ± 0.07† (60)	0.180 ± 0.05§ (69)
4	4.80 ± 1.40	3.20 ± 0.58 (33)	1.40 ± 0.35§ (71)	2.50 ± 0.75‡ (48)
5	17.40 ± 4.70	2.80 ± 0.66§ (84)	2.80 ± 0.42§ (84)	3.30 ± 1.00§ (81)
6	154.60 ± 53.90	42.70 ± 10.60§ (72)	35.80 ± 6.20§ (77)	33.00 ± 13.80§ (79)

[a] Mice were infected with 5×10^7 *M. lepraemurium* cells in the footpad. IL-2 was given 3 days (early courses) or 60 days (late courses) after the infection. In the early courses, mice received five injections of 400 U of IL-2 at 3-day intervals every month for 6 months, and in the late courses, they received a similar regimen of IL-2 for 4 months. The single shot was 20,000 U of IL-2 given on day 3 after infection. Untreated controls received saline. These data are summarized in Table 2. Difference from untreated controls: *, $P < 0.05$; ‡, $P < 0.02$; †, $P < 0.01$; §, $P < 0.001$.
[b] Absolute counts $\times 10^8$.
[c] Absolute counts $\times 10^6$.

untreated mice during the 6 months of the experiment (based on 10 to 15 mice per group; data not shown). Similarly, there was no significant mortality since mice do not die from *M. lepraemurium* infection until about 8 months after infection. IL-2 did not increase inflammation (swelling) at the site of *M. lepraemurium* infection, and in experiment 1 there was a small but significant reduction (Fig. 1c).

DISCUSSION

These results showed that IL-2 has a significant effect on bacterial cell counts after *M. lepraemurium* and BCG infection in mice. The effect on total bacterial numbers by

TABLE 2. Summary of effect of IL-2 on *M. lepraemurium* infection in BALB/c mice[a]

Expt	IL-2 treatment				Mean reduction in bacterial counts (%)		
	Dose (U)/shot	Shots/ course	Monthly courses	Time	Footpad	Lymph nodes	Liver
1	400	5	1	Early	10	71	66
2	400	5	6	Early	62	51	66
	400	5	4	Late	86	72	76
	20,000	SS	0	Early	61	60	67

[a] Mice infected with *M. lepraemurium* were given IL-2 on either day 3 (early) or day 60 (late) of infection. Experiments 1 and 2 are two separate experiments. The data are presented as the average of the mean percent reduction in bacterial counts at 4, 5, and 6 months as compared with the untreated controls. The detailed data of experiment 2 are shown in Table 1. SS, Single shot.

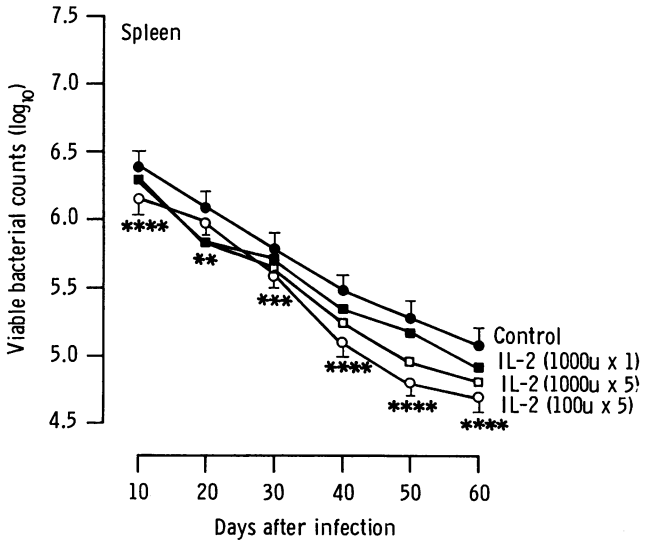

FIG. 2. Effect of IL-2 on viable bacterial counts in the spleen after BCG infection (experiment 1). Mice were infected intravenously with 5×10^7 BCG cells, and starting on day 3, they were given five daily injections of 100 or 1,000 U of IL-2 or a single injection of 1,000 U. The probability values are as described in the legend to Fig. 1 and are for mice given five injections of 100 U of IL-2 as compared with controls. Other details are described in the legend to Fig. 1.

TABLE 3. Summary of effect of IL-2 on BCG infection in BALB/c mice[a]

Expt	IL-2 treatment		Mean reduction in bacterial counts in the spleen (%)	
	Dose (U)/shot	Total injections	Total	Viable
1	100	5	39	50
	1,000	5	8[b]	41
	1,000	SS	15[b]	30
2	100	5	34	ND

[a] The data represent two separate experiments. IL-2 was given starting on day 3 after infection either once or daily for 5 days. In experiment 1 the average of the mean reduction in bacterial counts every 10 days from days 10 to 60 and in experiment 2 the weekly average from 7 to 42 days is shown. SS, Single shot; ND, not done.

[b] In these groups, there was a moderate early reduction of 27% (10 to 30 days) with a single shot of 100 U and 36% with five shots and an increase at later times (40 to 60 days).

microscopy is paralleled by a reduction in viable bacterial counts, indicating that IL-2 affects the viability (or replication) of organisms and not simply the degradation of dead organisms. With *M. lepraemurium*, the effect was seen in the liver, the regional lymph nodes, and also at the site of infection, except when a single course was given. There are several ways in which IL-2 may act. It may have a direct action on macrophages, and in fact, IL-2 is known to increase macrophage killing of tumor cells (14) and there is a preliminary report that IL-2 limits the replication of *Mycobacterium tuberculosis* in human macrophages (R. C. Bucklin and A. J. Crowle, J. Leukocyte Biol. **40**:324–325, 1986). Alternatively, IL-2 may act directly on macrophages through its ability to release gamma interferon from activated lymphocytes, as it is known that gamma interferon increases macrophage killing of mycobacteria (12, 19). It seems unlikely that IL-2 acts by activating NK or LAK cells since there is no evidence that these cells play a role in limiting bacterial as distinct from viral infection (20). However, NK cells do produce interferon and may be able to kill cells infected with mycobacteria. Finally, IL-2 may augment an effective immune response by inactivating suppressor cells. Our views on this effect are very much influenced by studies in the picryl (trinitrophenyl) chloride contact sensitivity system. Mice injected intravenously with spleen cells coated with haptene fail to develop contact sensitivity and actually become unresponsive and do not respond to formal immunization. However, these mice develop contact sensitivity when they are also given IL-2 on day 0 or 2 (5). Further experiments showed that the unresponsive population when treated with IL-2 in vitro behaves like immune cells on passive transfer. Detailed analyses have shown that these are I-J$^+$, Ly2$^+$ T suppressor cells (unpublished observations). However, it is pointed out here that there is no evidence yet for mycobacterial infections to show that IL-2 acts by inactivating suppressor cells.

The finding that IL-2 given late (2 months after infection) was more effective than IL-2 given early may have some practical significance. It suggests that IL-2 is of value in an established disease and hence of use in the treatment of human mycobacterial infection. Moreover, the dose of IL-2 used (400 U) is below the toxic level in humans when scaled up on a surface area or body weight basis (13). However, the reduction in bacterial counts was only approximately 60 to 70%, and repeated monthly courses did not increase the effect. In fact, with BCG, 100 U of IL-2 was actually more effective than 1,000 U. For this reason, IL-2 is unlikely to have a major therapeutic effect alone but may be of value when combined with other therapy. The paradox that higher doses of IL-2 have less effect than lower doses has also been described in other systems (20; R. C. Bucklin and A. J. Crowle, J. Leukocyte Biol. **40**:324–325, 1986).

Two approaches may be used to increase the therapeutic effect of IL-2. The first is to use IL-2 in combination with other lymphokines. In fact, a range of lymphokines are made after infection, and these agents often act synergistically: exposure to one agent increases the receptors for (and hence the effect of) other agents (18). This suggests that a combination of lymphokines may be more effective than IL-2 alone. The second approach is to use IL-2 with BCG vaccine for the treatment of established disease. The rationale is that certain mycobacteria limit the appearance of Ia on infected macrophages and hence interfere with antigen presentation (11). However, antigen-presenting cells newly arrived at the site of BCG vaccination may be normal. Hence, the combination of vaccination to provide mycobacterial antigen together with IL-2 to augment the immune response may be of value. These considerations justify investigation, in experimental animals, of the role of IL-2 together with BCG in the treatment of established mycobacterial diseases. Therapy of this type might in due course be valuable in human infections such as those produced by drug-resistant nontuberculous mycobacteria.

ACKNOWLEDGMENTS

A.J. thanks the Heiser Program for Research in Leprosy for the award of a postdoctoral fellowship.

We are grateful to John Farrant, Miroslav Malkovsky, and Brian Thomson for their constructive help and Peter Clark for his statistical assistance.

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