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Suppression of Immunity to *Mycobacterium lepraemurium* Infection

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After injection of 10^8 live *Mycobacterium lepraemurium* (MLM) into the left hind footpad of mice, there is development of local swelling attributable to a granuloma of the cell-mediated immunity type. Concomitant intravenous inoculation of live MLM delays and may even suppress footpad swelling, the effects being proportional to the intravenous dose of organisms. Concomitant footpad infection and intravenous inoculation of 10^9 dead MLM also delays footpad swelling, but over a period of months the feet become excessively swollen. The excessive swelling is due to local enhancement of infection as evidenced by an increase in the number of MLM per footpad. Attempts were made to prevent such immunosuppression by splenectomy or treatment with BCG. Splenectomy was entirely without effect, but 10^7 live BCG administered intravenously 2 to 4 weeks before dead MLM prevented enhancement of infection. The mediator of the immunosuppressive mechanism that results in enhanced infection remains to be elucidated, but it is unlikely to be antibody or immune complexes.

There are a number of infectious diseases, notably leprosy, with a wide spectrum of clinical forms. In leprosy, these may range from subclinical infection through tuberculoid and lepromatous forms (17). There is a close relationship between the differing clinical forms of leprosy and the state of cell-mediated immunity (CMI) to the antigens of *Mycobacterium leprae*. In tuberculoid leprosy there is a high level of CMI as evidenced by the positive Mitsuda and Fernandez reactions and the antigen-specific lymphocyte transformation reaction. Moreover, the pathology is characteristic of a hypersensitivity granuloma, and few viable *M. leprae* are present in the tissues. Lepromatous leprosy is noted for its lack of CMI to *M. leprae* antigens. The tissues of such patients are loaded with lepra bacilli, the cellular reaction to which notably lacks lymphocytes. Lepromatous patients are not tolerant to *M. leprae*, however, because their serum is rich in antibodies directed against the parasite.

A study of lepromatous disease has been initiated, using *M. lepraemurium* (MLM) infection of mice as the experimental model. Like *M. leprae*, this organism is an obligate intracellular parasite that invades almost every organ, grows within macrophages, and is remarkably nontoxic to its host. Like lepromatous patients, mice infected with MLM tolerate very large loads of bacilli, in excess of 10^{10}/mouse. The present investigation addressed the hypothesis that, by analogy with man, mice infected with MLM may develop CMI but are prone to lose it and thus develop a progressive, lepromatous infection. Based on the response of mice to sheep red blood cells (9, 12), it was postulated that CMI to MLM would be transitory or absent after i.v. infection, but might be more persistent after subcutaneous inoculation.

Since strains of mice differ widely in their susceptibility to MLM, it might be expected that CMI to MLM could be induced in resistant strains, and this has proved to be the case (3, 13). However, even a highly susceptible strain of mice generates a CMI response after footpad infection with MLM (M. Lefford et al., Infect. Immun., in press). This paper is concerned with the specific suppression of that response by a concurrent systemic injection of homologous antigen and the repercussions it has on the course of local infection in the footpad.

MATERIALS AND METHODS

Mice. Female (BALB/c × C57BL/6)F_1 mice (CB6) were bred at Trudeau Institute, Inc., Saranac Lake, N.Y., and admitted to experiments when 6 weeks old.

MLM. The Hawaii strain of MLM was passaged in CB6 mice, periodically harvested from the liver and spleen, and purified by a modified Draper procedure (5). Portions of bacterial suspension of known density were stored at −70°C and recovered immediately before use. A separate batch of MLM was heated to 100°C for 15 min, distributed into vials, and stored at −70°C. This material was designated as heat-killed MLM (HK-MLM) and was shown to be noninfectious to mice.

BCG. The Pasteur strain of BCG was obtained

from the Trudeau Mycobacterial Culture Collection (TMC 1011), grown in Proskauer and Beck medium containing glycerol and Tween 80, and stored at −70°C. The viability of the culture was known before use, and 10^7 live organisms in 0.2 ml of saline diluent were injected intravenously (i.v.).

Immunization and challenge with MLM. Mice were immunized by injecting 10^8 live MLM in a volume of 0.04 ml of culture medium into the left hind footpad (LHFP). Appropriate numbers of live or HK-MLM were suspended in 0.2 ml of culture medium for i.v. challenge.

Splenectomy. Spleens were removed from mice under ether anesthesia through a left subcostal incision. The skin was closed with Michel clips. Sham-splenectomized mice were similarly treated except that the spleen was not excised. Mice were allowed to recover for 8 weeks after surgery before being admitted to an experiment.

Organ measurements. Hind-footpad thickness was measured with dial gauge calipers. The difference in thickness between left and right hind feet was expressed in 0.1-mm units. Spleens were weighed with a top-loading balance.

Counts of MLM. LHFP were homogenized in saline by using a VirTis grinder. The larger fragments of skin and bone were allowed to settle, and appropriate dilutions of the supernatant were used to prepare smears on Reich slides (15). The slides were stained with phenol auramine-rhodamine and scanned with a fluorescence microscope (18). The stained organisms were counted, and estimates of the number of MLM per LHFP were calculated. Preliminary experiments revealed that smears of living and HK-MLM yielded identical counts when stained with auramine-rhodamine or carbol fuchsin.

Statistics. Footpad thickness and spleen weight data were in arithmetic units, but the number of MLM per LHFP were expressed geometrically as $\log_{10}$. All data were evaluated by analysis of variance. Comparisons of group means were made by using either the Q test (16) or the Dunnett test (1), as appropriate.

RESULTS

Concomitant intravenous and footpad infection. Mice were infected with 10^8 live MLM into the LHFP. On the same day, they were divided into groups of 10, which were given either 10^9, 10^8, or 10^7 live MLM i.v., while a control group received no i.v. challenge. The increase in LHFP thickness was measured at intervals for up to 16 weeks (Fig. 1). Within 2 weeks of footpad infection, the LHFP of the control mice swelled substantially and continued to do so until the 6th week, after which the footpad size remained fairly steady. Footpad swelling was almost entirely suppressed in mice receiving 10^9 live MLM i.v. and was substantially delayed in the mice given 10^8 MLM i.v. The 10^7 dose was the least disturbing, but even so the LHFP thickness was half that of the controls at 3 weeks. The mice given 10^9, 10^8, and 10^7 live

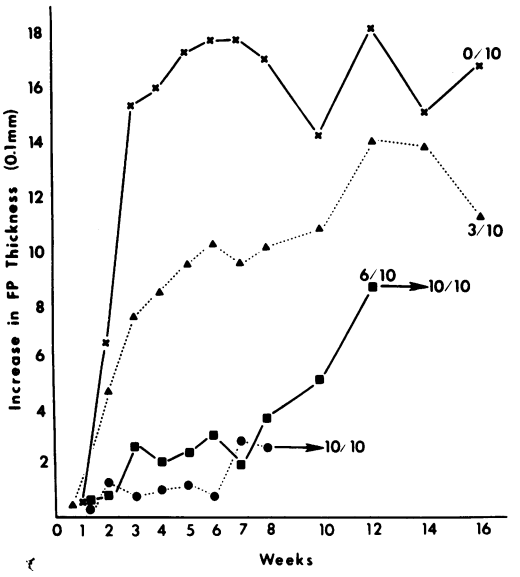

FIG. 1. *Effect of concomitant i.v. infection on footpad infection with 10^8 MLM. Symbols: ×, no i.v. infection; ▲, 10^7 MLM i.v.; ■, 10^8 MLM i.v.; and ●, 10^9 MLM i.v. The numbers on the chart denote dead mice/total mice.*

MLM i.v. had all died by weeks 10, 14, and 18, respectively. The control mice, which had been infected in the footpad only, survived for 36 weeks. Since it has previously been shown that swelling at the infection site is due to granuloma formation after induction of CMI (M. Lefford et al., in press), this experiment was tentatively interpreted as demonstrating the suppression of CMI by i.v. infection with MLM.

Effect of HK-MLM on the immune response to live MLM. (i) Concomitant challenge. Groups of 10 mice each were infected with 10^8 live MLM into the LHFP and, on the same day, were given either 10^9 live MLM, 10^9 HK-MLM, or saline i.v. An additional group of mice was challenged with 10^9 HK-MLM i.v. not only on the day of infection but at weekly intervals for 6 weeks. As before, the injection of 10^9 live MLM i.v. almost completely suppressed LHFP swelling, and the mice thus infected died within 14 weeks (Fig. 2). The single dose of 10^9 heat-killed organisms caused a delay in the onset of footpad swelling by only 1 week, but repeated inoculations of antigen suppressed the response for several weeks longer. Until the 10th week of the experiment, it appeared that the effect of HK-MLM i.v. was merely to delay the immune response without affecting it qualitatively. However, beyond that point, the LHFP swelling of mice given HK-MLM i.v. continued to increase,

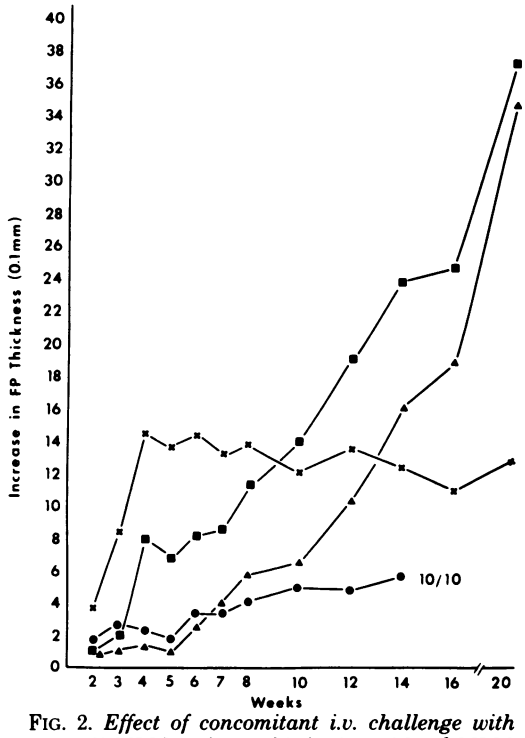

FIG. 2. *Effect of concomitant i.v. challenge with live or HK-MLM on footpad infection with 10^8 MLM. Symbols: ×, no i.v. infection; ■, 10^9 HK-MLM i.v. at week 0; ▲, 10^9 HK-MLM at weeks 0 to 6; and ●, 10^9 live MLM i.v. at week 0. Numbers on the chart denote dead mice/total mice.*

whereas that of control animals had leveled off.

All mice were observed until 32 weeks, when they began to die. At that time, the LHFP of mice that had received HK-MLM were extremely turgid and ulcerated (Fig. 3). This raised the question of whether excessive swelling represented an exaggerated CMI reaction or was a local enhancement of infection with the features of a leproma. Accordingly, four mice of each surviving group were killed, and counts of MLM per LHFP were made. The mean counts per LHFP were 2.5×10^9 for controls, 3.6×10^{10} for mice given a single i.v. dose of HK-MLM, and 4.9×10^{10} for mice given repeated i.v. injections of HK-MLM. The first value was significantly lower ($P < 0.01$) than the latter two, which did not differ significantly from each other.

(ii) Immunization with HK-MLM before infection. The effect of i.v. immunization with HK-MLM before the initiation of footpad infection was examined next. Groups of 10 mice each were given 10^9 HK-MLM i.v. either 8, 6, 4, 2, or 0 weeks before 10^8 live MLM was injected into the LHFP. Another group was given 10^9 HK-MLM i.v. repeatedly at weeks −8, −6, −4, −2, and 0. Finally, there were additional groups that received either no i.v. challenge or 10^9 live MLM i.v. at the time of footpad infection. The increases in footpad swelling at the infection site are represented in Fig. 4. Once again the concurrent i.v. infection suppressed the footpad response and killed the animals within 12 weeks. Among the mice receiving HK-MLM i.v., the most marked delay in the onset of footpad thickening occurred in those that had been hyperimmunized or given a single dose at the time of infection. More remarkable than the delay in the onset of the response was the ultimate increased swelling in the LHFP in recipients of HK-MLM i.v. All the groups of mice that had received HK-MLM i.v. had LHFP that were more swollen than those of the controls at week 20, but the increased swelling was statistically significant ($P < 0.01$) only for the mice that had been hyperimmunized or had received HK-MLM 4 weeks before or at the time of footpad infection.

At 20 weeks, four mice per group were randomly selected, and their spleen weights and number of MLM per LHFP were determined (Table 1). All groups that had received HK-MLM i.v. had significantly higher ($P < 0.01$)

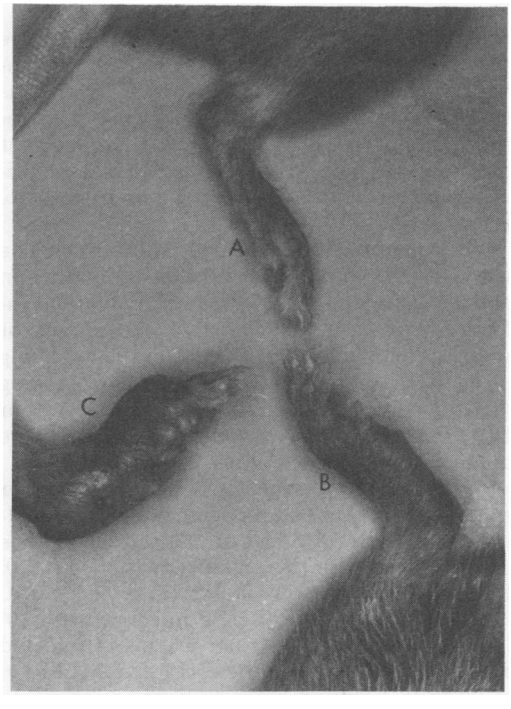

FIG. 3. *Swelling of left hind footpad 20 weeks after infection with 10^8 MLM. (A) Normal uninfected foot; (B) 10^8 MLM LHFP and no i.v. challenge; and (C) 10^8 MLM LHFP and concomitant challenge with 10^9 HK-MLM i.v.*

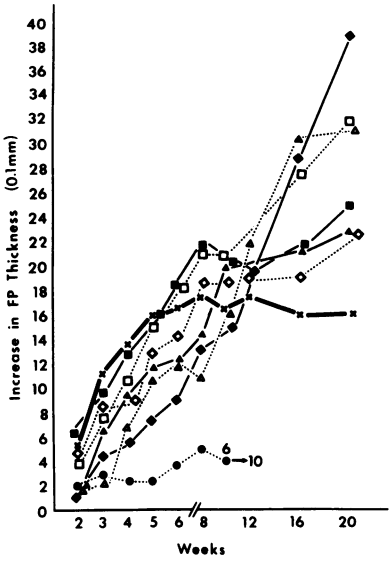

FIG. 4. *Effect of immunization with HK-MLM on footpad infection with 10^8 MLM. Symbols; ×, no i.v. challenge; ●, 10^9 live MLM i.v. at week 0; 10^9 HK-MLM i.v. at either week 0 (△), −2 (▲), −4 (□), −6 (■), −8 (◇), or −8, −6, −4, −2, and 0 (◆). The numbers on the chart signify dead mice per group of 10.*

counts of MLM in the LHFP than those mice that had not been challenged i.v., denoting enhancement of infection in the former. There was considerable variation between groups with respect to spleen weight, but only the mice that had received HK-MLM on day 0 had significantly larger ($P < 0.01$) spleens than the control group.

Prevention of enhanced infection. The above experiments indicated that the induction and/or expression of CMI to MLM footpad infection had been blocked by concomitant or prior i.v. challenge with MLM antigens, living or dead. In another system, analogous blocking of CMI could be alleviated by splenectomy or BCG infection (9, 10), so these procedures were applied in the present study.

(i) Effect of splenectomy. Mice were either splenectomized or sham-splenectomized and allowed to recover for 8 weeks. Groups of 10 mice of each type were then challenged i.v. with 10^9 HK-MLM at either week −2, 0, or weeks 0, 1, 2, 3, and 4, relative to LHFP infection with 10^8 live MLM at week 0. Other groups of infected mice received either saline or 10^9 live MLM i.v. at week 0. Footpad measurements were made at regular intervals for 20 weeks (Fig. 5). The data from some experimental groups, namely those given HK-MLM at week −2 and weeks 0 to 4, are omitted from this figure for the sake of clarity. These groups yielded essentially similar results to the similarly treated groups represented in Fig. 4 and 2, respectively.

Considering first the sham-splenectomized mice, the results are closely similar to those obtained previously: 10^9 live MLM i.v. suppressed footpad swelling and was lethal; 10^9 HK-MLM i.v. delayed the onset of footpad swelling, which later exceeded that in unchallenged controls. The effects of splenectomy were trivial. There was slightly increased footpad swelling in

TABLE 1. *Effect of pretreatment with HK-MLM i.v. on footpad infection with MLM*[a]

Pretreatment with 10^9 HK-MLM i.v. at week[b]:	Increase in footpad thickness (0.1 mm)	No. of MLM per LHFP ($\log_{10}$)	Spleen wt (mg)
No pretreatment[c]	14.5	9.14	235
0	32.2[d]	10.09[d]	489[d]
−2	23.8	10.03[d]	142
−4	30.0[d]	10.29[d]	105
−6	26.0	10.12[d]	167
−8	20.5	10.03[d]	152
−8, −6, −4, −2, and 0	34.8[d]	10.36[d]	320

[a] Results obtained 20 weeks after infection.
[b] Relative to infection with MLM at week 0.
[c] Group against which all statistical comparisons were made.
[d] $P < 0.01$.

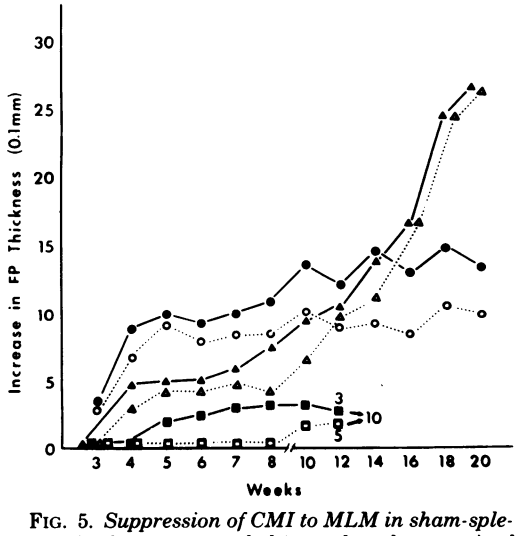

FIG. 5. *Suppression of CMI to MLM in sham-splenectomized (open symbols) and splenectomized (closed symbols) mice. All mice received 10^8 live MLM per LHFP at week 0. On the same day, the mice were given either no i.v. challenge (circles), 10^9 live MLM i.v. (squares), or 10^9 HK-MLM i.v. (triangles). The numbers on the chart denotes the dead mice per group of 10.*

all groups of splenectomized mice, but survival of mice given 10^9 live MLM i.v. was not prolonged and enhancement of footpad swelling in mice that had received HK-MLM was not prevented. At week 20, four mice from each group were selected at random and the number of MLM per LHFP was counted (Table 2). Intravenous challenge with HK-MLM induced highly significant ($P < 0.01$) increases in footpad thickness and the number of MLM at the infection site in sham-splenectomized and splenectomized mice, and there was no statistically significant difference between them. Multiple injections of HK-MLM produced no greater degree of footpad swelling or increase in the number of MLM per LHFP than a single dose ($P > 0.05$).

(ii) Effect of BCG on i.v. challenge with live MLM. Groups of 10 mice each were given 10^7 live BCG at either week -4, -3, -2, -1, or 0, relative to injection of 10^8 live MLM into the LHFP. On the day of footpad infection these mice were challenged with 10^9 live MLM i.v. (Table 3). Footpad measurements (not shown) were made weekly, but substantial swelling occurred solely in mice that had received only 10^8 live MLM into the footpad. BCG had no sparing effect on the suppressive effects of i.v. MLM upon footpad swelling. Furthermore, it is clear from the data in Table 3 that BCG did not significantly prolong the survival of challenged mice.

(iii) Effect of BCG on i.v. challenge with HK-MLM. Groups of mice were immunized with 10^7 live BCG i.v. on week -4, -3, -2, -1, or 0, relative to footpad infection with 10^8 live MLM. Additional groups of mice received no BCG. Control and BCG-immunized mice were challenged with 10^9 HK-MLM i.v. on weeks 0, 1, 2, 3, and 4 (total dose of 5×10^9 organisms per mouse), and footpad measurements were made for 22 weeks (Fig. 6). It first appeared that BCG had no effect on the suppressive action of HK-MLM in that the onset of footpad swelling was delayed and subequently exceeded that of controls. However, by 22 weeks, the swelling of the LHFP of BCG- and HK-MLM-immunized mice (groups 3 to 7) was less than the grossly swollen feet of mice that had received HK-MLM i.v. but no BCG (group 2), yet greater than the modest thickening observed in mice that had been given neither HK-MLM nor BCG (group 1). At this point, four mice per group were randomly chosen, and viable counts of the MLM per LHFP were made (Table 4).

Only the left hind feet of mice in groups 2 and 3 were significantly thicker than those of mice in group 1. However, the feet of mice in group 2 were significantly more swollen than those of mice in any other group ($P < 0.05$ to 0.01). Similarly, only group 2 mice had significantly greater numbers of MLM per LHFP than group 1. However, groups 5, 6 ($P < 0.05$), and 7 ($P < 0.01$) had significantly fewer MLM per LHFP than group 2. In essence, the treatment with BCG had restored groups 5 to 7 to the status of group 1 by negating the infection-enhancing effects of HK-MLM. It is notable that BCG given at the time of HK-MLM or 1 week earlier (groups 3 and 4) was much less effective in this regard.

TABLE 2. *Effect of splenectomy upon enhancement of footpad infection with HK-MLM i.v.*

Challenge with 10^9 HK-MLM i.v. at week[a]:	Sham-splenectomy		Splenectomy	
	Increase in size of LHFP (0.1 mm)	No. of MLM per LHFP ($\log_{10}$)	Increase in size of LHFP (0.1 mm)	No. of MLM per LHFP ($\log_{10}$)
No challenge	7.2	8.31	14.0	8.54
−2	27.0[b]	9.73[b]	36.2[b]	9.92[b]
0	24.7[b]	9.64[b]	23.8[c]	9.89[b]
0 to 4	30.5[b]	9.93[b]	30.7[b]	9.79[b]

[a] Relative to infection with MLM at week 0.
[b] $P < 0.01$.
[c] $P < 0.05$.

TABLE 3. *Effect of pretreatment with living BCG on mortality after infection with 10^9 living MLM i.v.*

Pretreatment with 10^7 BCG i.v. at week[a]:	Infection with:		Survivors (out of 10)				
	10^8 MLM LHFP	10^9 MLM i.v.	11[b]	12	13	14	15
No pretreatment	+	No infection	10	10	10	10	10
No pretreatment	+	+	10	3	1	0	
0	+	+	10	4	2	0	
−1	+	+	10	9	4	2	0
−2	+	+	10	9	4	3	0
−3	+	+	10	8	5	0	
−4	+	+	10	10	3	0	

[a] Relative to infection with MLM at week 0.
[b] Weeks after MLM infection.

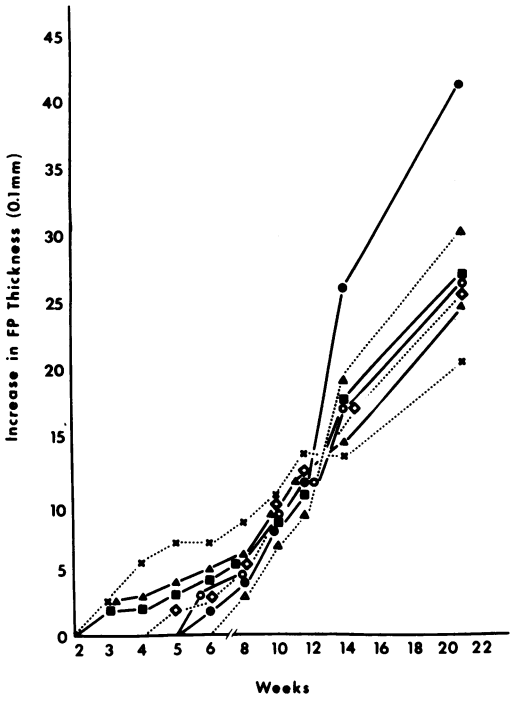

FIG. 6. *Effect of BCG on suppression of CMI to MLM. 10^7 BCG i.v. was given either −4 (▲), −3 (■), −2 (◇), −1 (○), or 0 (△) weeks relative to 10^8 MLM in the LHFP. Two MLM-infected groups received no BCG (×, ●). All but one group (×) were challenged with 10^9 HK-MLM i.v. at weeks 0 to 4.*

DISCUSSION

For some years there has been interest in the factors that determine the induction of CMI, its duration, and decay. Most of these studies have used commonplace inanimate antigens, such as haptens, serum proteins, or heterologous erythrocytes, and only one manifestation of CMI has been evaluated, delayed-type hypersensitivity. Only recently has much effort been devoted to systems involving viable replicating antigens, namely tumors. It has been found that many transplantable tumors induce a state of CMI that may be subverted either spontaneously, during the growth of the tumor, or by preimmunization with homologous antigens. Suppression of CMI enhances the growth of the tumor. Such enhancement has been ascribed to a variety of "blocking" agents: excess circulating antigen, excess circulating antibody, immune complexes (2, 7, 8), or suppressor cells (6).

By analogy with the tumor models, it was postulated that similar "blocking" mechanisms might exist in infectious diseases that are characterized by chronicity and progression in the face of an initial CMI response. Human leprosy appeared to be one such disease, and its experimental analog, MLM infection of mice, has been investigated from this viewpoint.

CB6 mice infected with MLM in the footpad develop CMI, one manifestation of which is a granulomatous swelling of the footpad. In this study, the kinetics of footpad swelling was used as a marker of the induction of CMI to MLM. It was found that footpad swelling was substantially delayed by concurrent i.v. infection with MLM, the extent of delay being proportional to the size of the i.v. challenge. Live MLM (10^9) i.v. produced virtually complete suppression of footpad swelling until the mice died. The late effects of concurrent i.v. antigen on footpad infection were not seen, however, because the mice died of systemic disease. But when mice were injected i.v. with HK-MLM, it became clear that there were both early and late effects. The early effect comprised a delay in the onset of footpad swelling. A single injection of 10^9 HK-MLM i.v. on the day of infection caused a minimal delay of footpad thickening, similar to that engendered by 10^7 live MLM i.v. The delay could be protracted by repeated doses of antigen during the induction period of CMI. The second consequence of i.v. antigen was a late and massive increase in footpad swelling above that observed in appropriate controls. This was not seen until the fourth month of infection. It seemed that the excessive swelling might be attributed either to a delayed but exaggerated CMI response or to enhancement of bacterial growth in the footpad. Bacterial counts revealed that the latter alternative was correct. The experiments in

TABLE 4. *Prevention of enhancement of MLM infection by BCG*[a]

Group	Immunization with 10^7 BCG i.v. at week[b]:	Challenge with HK-MLM i.v.	Swelling of LHFP (0.1 mm)	No. of MLM per LHFP ($\log_{10}$)
1	No immunization	−	20.5	9.63
2	No immunization	+	42.1[c]	10.38[c]
3	0	+	30.3[d]	10.16
4	−1	+	26.4	10.15
5	−2	+	25.8	9.65
6	−3	+	27.1	9.57
7	−4	+	25.2	9.29

[a] Footpad swelling and MLM content at 22 weeks postinfection.
[b] Relative to infection with MLM at week 0.
[c] $P < 0.01$.
[d] $P < 0.05$.

which live or dead MLM were given i.v. on the day of footpad infection are analogous to the experiments of Lagrange et al. (9), in which suppression of the CMI response to a peripheral injection of sheep erythrocytes was suppressed by an i.v. injection of sheep erythrocytes.

The ability of HK-MLM to modify MLM infection was not confined to challenge at the time of infection. Pre-immunization with HK-MLM as early as 8 weeks before infection enhanced that infection. It is noteworthy that pre-immunization produced no discernible delay in the onset of footpad swelling, but had a substantial influence on the eventual size of the foot. By analogy with other studies (9, 11), it was thought that suppression of CMI and consequent enhancement of infection might be caused by blocking antibodies or immune complexes (2, 7, 8). Since antibody production after i.v. immunization with particulate antigens is largely dependent on the presence of the spleen, the effect of splenectomy was of great interest (14). As it happened, the splenectomy was entirely without effect on the suppressive action of MLM i.v., suggesting that neither antibodies nor immune complexes were involved or that the spleen plays a less dominant role in the humoral response to this highly persistent antigen.

Another device that had been found useful in the prevention of blocking CMI by an excessive antigenic stimulus was BCG infection (10). In this system, BCG failed to influence the blocking action of live MLM i.v. However, BCG given 2 to 4 weeks before infection ameliorated the effects of HK-MLM in that excessive footpad swelling was reduced and multiplication of MLM in the footpad was no greater than in normal mice.

The action of BCG as an immunomodulating agent is complex and poorly understood, even with respect to unrelated antigens. This complexity is compounded in the case of another mycobacterial infection in which cross-reactivity exists between mycobacterial species (4). Certainly it would be unjustifiable to conclude, based on the present evidence, that BCG functions in the same way in both the MLM and sheep erythrocyte systems. The evidence presented here casts some doubt on the relevance of humoral factors in the regulation of the CMI response to MLM infection. Experiments in progress are being directed to examine this problem in greater detail, with particular emphasis on nonhumoral factors such as suppressor cells (6).

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