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Lymphocytic Choriomeningitis Virus-Induced Immunosuppression: Evidence for Viral Interference with T-Cell Maturation

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Acute lymphocytic choriomeningitis virus (LCMV) infection is associated with general immunosuppression which develops during the second week of the infection and persists for several weeks. In the present study, the ability of LCMV-infected mice to mount a cytotoxic T-lymphocyte response was investigated in a transplantation assay, using LCMV-immunized mice as recipients. By this means it was possible to evaluate the T-cell responsiveness of the acutely infected mice separately. Our results revealed a marked depression of the T-cell function temporally related to immunosuppression in the intact mouse. Furthermore, this hyporesponsiveness could not be explained as an effect of suppressor cells. Occurring shortly before these changes were a drastic decrease in cortical thymocytes and a reduction in T-cell progenitors in the bone marrow and spleen. Our findings are consistent with the assumption that a numerical deficiency of immunocompetent T-cells due to viral interference with T-cell maturation plays an important role in LCMV-induced immunosuppression.

Primary, nonfatal lymphocytic choriomeningitis virus (LCMV) infection of immunocompetent mice, although resulting in a potent virus-specific immune response and subsequent virus clearance, is associated with prolonged, but transient, depression of immune responsiveness to a variety of antigens. This viral immunosuppression was first described by Mims and Wainwright, who showed reduced humoral immune responses to sheep erythrocytes and human serum albumin (18). Bro-Jørgensen and colleagues extended these findings, and their results indicated that humoral responses to thymus-dependent antigens were selectively affected (5, 8).

Depressed cellular immunity was first suggested by a decreased ability of mice acutely infected with LCMV to mount footpad swelling to ectromelia virus and an increased susceptibility to challenge with this virus (18). A delayed rejection of skin (16) and tumor allografts (12) and a depressed splenic cytotoxic T-lymphocyte (CTL) response to alloantigen (12) have provided confirming evidence.

Despite these findings demonstrating clear-cut depression of cell-mediated immunity, the underlying cellular mechanisms remain obscure. It is notable that reduced in vitro mitogen responsiveness during acute infection can be restored by the addition of 2-mercaptoethanol or normal peritoneal macrophages (14). Although this restorative effect suggests some defect in the macrophage population of the infected spleen, it does not give definitive proof of failure of the in vivo function of macrophages. One way to differentiate between a defect at the level of the T-cells and the antigen-presenting splenic environment is to stimulate cells from acutely infected donors in uninfected lethally irradiated recipients. Since the uninfected recipients would constitute the antigen-presenting environment, the responsiveness of the donor T-cells should be evaluated separately (9). Using an assay of this design, we observed that the CTL response resulting from transfer into irradiated F_1 hybrids was markedly reduced when the responding parental T-cells came from LCMV-infected mice, and most importantly, it was ascertained that this impaired response was not an artifact caused by carry-over of the virus with the donor cells. Moreover, it was clearly demonstrated that the hyporesponsiveness could not be explained as a conceivable effect of suppressor cells. The results, therefore, indicate a numerical deficiency in at least one of the T-cell subsets collaborating in the generation of CTL. This depletion is temporally related to a previously demonstrated reduction of the T-helper cell population for humoral responses (5, 8). As this suggested a common mechanism for the deficiency of immunocompetent T-cells, further efforts were made to study T-cell maturation and,

in particular, changes in the T-cell progenitor pool of the bone marrow and spleen during acute infection. Our results support the assumption that immunosuppression is closely related to the general dysfunction of the hemopoietic stem cells, which is also seen during acute LCMV infection (4), a hypothesis recently contested by others (22).

MATERIALS AND METHODS

Virus. LCMV of the viscerotropic Traub strain was used throughout the study. The virus preparations used were tissue culture supernatants obtained from monolayers of infected L-cells. The stock virus was recently examined for viral contamination, and none of the 11 murine contaminants described by Collins and Parker (10) were found, nor were ectromelia and cytomegalovirus found. The preparations were kept at −70°C. Virus titrations were carried out by intracerebral inoculation into young adult Swiss mice; administered by this route, LCMV produces a fatal disease. Titration endpoints were calculated by the Kärber method and expressed as mean lethal doses (LD_{50}) per 0.03 ml.

Mice. The effect of LCMV infection was studied in C3H/Ssc/1 mice 2 to 4 months of age. Acute infection of adult mice was produced by intraperitoneal (i.p.) inoculation of 1,000 times the LD_{50} of the virus; this procedure causes nonfatal, transient, and immunizing infection. Immune mice to be used as recipients were taken 2 to 4 months after the infection. Adult LCMV carrier mice had been inoculated within 18 h of birth with 1,000 times the LD_{50} of the virus. As previously described (27), this treatment results in a state of immunological tolerance, and the mice carry the virus in high titers in the blood and organs throughout life. For activation of alloreactive CTL, lethally irradiated F_1 hybrids (C3H × DBA/2) were used as recipients.

X-Irradiation. Irradiation was administered by a Siemens Stabilipan therapy machine operated at 200 kV and 15 mA, with 1.0-mm copper filtration. The dose rate was 47 R/min, and the half-value layer was 1.5-mm copper.

Preparation of cells. For estimation of cellularity, cytotoxicity assay, and transplantation purposes, cell suspensions from spleens, thymuses, or femoral bone marrow were prepared in Hanks balanced salt solution and counted as previously described (26). Transplantations were done with volumes of 0.5 ml and were always between animals of the same sex.

Target cells. Tumor lines carrying the alloantigens of DBA/2 ($H\text{-}2^d$; P815 mastocytoma) and C57BL/6 ($H\text{-}2^b$; EL-4 lymphoma) were kindly provided by Flemming Güttler of this institute and were propagated both in vivo and in vitro.

Assay for cytotoxic cells. The procedure was essentially the same as that previously described (24), except that allogeneic tumor cells were used as targets. To measure the relative number of cytotoxic cells present in any suspension, serial twofold dilutions of effector cells were mixed with a fixed number of ^{51}Cr-labeled targets to obtain no less than four effector/target ratios (usually 30:1, 15:1, 7.5:1, and 3.8:1). After incubation at 37°C for 15 to 16 h, the cell-free supernatants were collected for measurement of the radioactivity released. Percent specific release was calculated according to the formula: (experimental − spontaneous release)/(water − spontaneous release) × 100. From these data the number of effectors corresponding to 50% specific release (one LU_{50}) was determined graphically. Very low responses were approximated, as recently described, and expressed as less than the calculated value (24). The number of LU_{50} per spleen was then calculated from the total number of cells yielded by each spleen (average calculated from three to four pooled spleens).

Assay for immunocompetent T-cells. To assay for immunocompetent T-cells, a fixed number of spleen cells prepared from a pool of at least four donor spleens were transferred intravenously into groups of 3 to 4 F_1 hybrids, which had been X-irradiated with 800 R 2 to 3 h previously (9). Four days later, the recipients were sacrificed, and the spleens of each experimental group were pooled. The total cell yield per spleen was determined, and samples of the single-cell suspensions were assayed for cytotoxic cells as described above. The total cytotoxic activity per recipient spleen was taken as a measure of the relative level of immunocompetent T-cells in the number of cells transferred (20). Preliminary titration experiments ensured that the cell dose chosen (20×10^6) was within the dose range, giving approximate proportionality between cell dose and total cytotoxic activity.

Assay for T-cell progenitors. It is generally accepted that only T-cell progenitors repopulate the thymus of a lethally irradiated, marrow cell, or spleen cell-reconstituted recipient (15, 17). The assay for cells with thymus-repopulating capacity was performed essentially as described by Tyan (25), with the important modification that either LCMV-immunized C3H mice or C3H virus carriers were used as recipients to avoid undesirable effects of possible virus contamination of the donor cells. Briefly, recipients (five mice per experimental group) were given 800 R of whole body X-irradiation and, within 3 h, were injected intravenously with 3×10^6 femoral bone marrow cells or 25×10^6 spleen cells prepared from pooled organs of three to four infected C3H mice or uninfected controls. Twenty days later the recipients were killed, and the weight, and cellularity of the thymuses were determined individually.

Thymus histology. Specimens were fixed in a 5% aqueous formaldehyde solution and stained with hematoxylin and eosin.

RESULTS

LCMV-induced changes in the peripheral T-cell compartment. To separate a direct effect of LCMV on the T-cell populations involved in the alloreactive CTL response from effects secondary to a virus-induced defect of the antigen-presenting environment, normal spleen cells and spleen cells from infected mice were compared with regard to the formation of CTL after transfer into lethally irradiated F_1 hybrids. In this assay, however, recipients not immunized to LCMV would themselves undergo acute infection due to carry-over of the virus from the donors, thereby suppressing the response (data

TABLE 1. The influence of virus inoculation upon the ability of 800-R-irradiated LCMV-immunized C3H × DBA/2 mice to support activation of alloreactive CTL from C3H mice

Recipient mice	Inoculation of virus[a]	LU_{50} per spleen against:[b]	
		P815 (H-2^d)	EL-4(H-2^b)
Immune[c]	nil	118	0
Immune	10^5 LD_{50}	82	0
Normal	nil	81	0

[a] Virus was inoculated i.p. at the time of cell transfer.

[b] Cytoxicity against DBA/2 (H-2^d) and C57BL/6 (H-2^b) tumor cells 4 days after transfer of 20 × 10^6 C3H spleen cells.

[c] Immunized with 10^3 times the LD_{50} i.p. 3 months previously.

TABLE 2. Alloreactive CTL responses of spleen cells from infected C3H mice after transfer to 800-R-irradiated LCMV-immunized C3H × DBA/2 mice

Donor mice	No. of cells per donor spleen (×10^6)	LU_{50} per recipient spleen[a]	Donor CTL potential	
			LU_{50}[b]	%[c]
Normal	102	49	248	100
Infected (day 4)[d]	98.5	72	354	143
Infected (day 9)	92.5	<15	<68	<27
Infected (day 14)	325	<3	<52	<21
Infected (day 20)	175	16	140	57
Infected (day 30)	108	53	287	116

[a] Cytotoxicity against DBA/2 tumor cells 4 days after transfer of 20 × 10^6 C3H spleen cells.

[b] Relative CTL response corrected for total cell number of donor spleen.

[c] Percentage of normal.

[d] Inoculated with 10^3 times the LD_{50} of LCMV i.p. on day zero.

not shown). Therefore, immunized recipients were used in which deliberate virus inoculation was shown not to interfere with the generation of CTL (Table 1).

When the immune responsiveness was assessed under these conditions (Table 2), responses of equal magnitude were obtained with normal donor cells and cells from mice infected 4 days previously. However, when a similar number of cells came from mice infected 9, 14, or 20 days earlier, the generation of CTL in irradiated F_1 mice was found to be significantly reduced. Even when compensation was made for the changes in spleen cellularity during acute infection, LCMV-infected mice appeared clearly deficient compared with uninfected animals. Normal immunocompetence was not restored until about 1 month after the infection.

The results obtained could be explained on the basis of one (or both) of the following assumptions: a deficiency of immunocompetent T-cells or the presence of suppressor cells. In an attempt to demonstrate the presence of suppressor cells, we carried out the following experiment. Pooled spleen cell suspensions were prepared from normal mice and from mice inoculated with LCMV 13 days earlier. Then three cell suspensions were made which contained the following cell doses per 0.5 ml: (i) standard dose of normal cells, (ii) standard dose of suppressed cells, and (iii) the standard doses of both normal and suppressed cells. Each of three groups of lethally irradiated recipients received one of these suspensions. From the data recorded in Table 3 it appears that donor cells from infected mice gave rise to a reduced response and, moreover, that the CTL response achieved with the mixture of infected and normal cells was close to the sum of the responses produced by the separate cell suspensions. The results, therefore, made it unlikely that suppressor cells (or carry-over of virus) played any significant role for the suppressed adoptive responses seen in recipients of cells from infected donors.

LCMV-induced changes in the thymus. By 4 to 7 days after i.p. infection with the Traub strain employed in this study, the number of thymocytes was halved. Histologically, the medulla appeared normal, whereas there was depletion of cortical lymphocytes, starting as "patchy areas" and progressing into diffuse depletion. At day 10 postinfection (p.i.), the total cell number was reduced to 15% of the original, and cortical lymphocyte depletion was almost complete, leaving large pale reticular cells. No changes were found in the medulla even at this time. By day 19 p.i., the number of thymocytes was still markedly reduced; but by day 24 p.i., thymus cellularity and structure were restored to normal. It was notable, and in contrast to others demonstrating LCMV-induced thymic involution (13), that we did not find signs of cell destruction on any day tested, indicating that increased destruction of lymphocytes within the thymus played no major role in the reduction of thymocytes.

LCMV-induced changes in the T-cell progenitor pool. The following experiments were aimed at evaluating the capacity of progenitor cells from the LCMV-infected mice to restore the thymuses of lethally irradiated recipients. Previous results have shown a complete abolition of stem cell function in acutely infected recipients (7), which would consequently die before significant restoration of the thymus; thus, immunized mice were chosen as recipients. Experiments showed that deliberate inoculation of virus to this type of recipient did not interfere with the repopulation of the thymus (Table 4), indicating that the restorative capacity of virus-contam-

TABLE 3. Interaction between LCMV-suppressed and normal C3H spleen cells in the alloreactive CTL response after transfer to 800-R-irradiated LCMV-immunized C3H × DBA/2 mice

Donor cells	CTL response per recipient spleen[a]	
	LU_{50}	%
Normal (20×10^6)	68	100
Suppressed (20×10^6)[b]	<4	<6
Normal (20×10^6) + Suppressed (20×10^6)	96	141

[a] Cytotoxicity against DBA/2 tumor cells 4 days after cell transfer.

[b] Spleen cells taken 13 days after i.p. inoculation of 10^3 times the LD_{50} of LCMV.

inated cells could be assessed with confidence.

Table 4 shows the changes in T-cell progenitors as measured by transfer to such recipients. In the bone marrow, the thymus-restoring capacity dropped promptly during the very first days after virus inoculation and remained markedly depressed for at least 3 weeks. In the spleen, the thymus-restoring capacity was significantly reduced on day 4 p.i., it was normal on day 8 p.i., and it seemingly increased above normal on day 13 p.i. That carry-over of virus did not play any significant role in these findings was further substantiated by the observation that essentially similar results were obtained by using LCMV virus carrier mice as recipients (Table 4).

DISCUSSION

It has previously been demonstrated that the splenic CTL response to allogeneic tumors is markedly reduced in acutely LCMV-infected mice (12). In the present study, the ability to generate an allospecific CTL response during acute infection was examined in a transplantation assay, using lethally irradiated LCMV-immunized recipients to prevent any effect of the virus upon the antigen-presenting environment (Table 1). Therefore, the hyporesponsiveness demonstrated (Table 2) indicates a defect residing within the immunocompetent T-cell population. The experiment assessing interaction between LCMV-suppressed and normal spleen cells (Table 3) makes it unlikely that suppressor cells (or carry-over of virus) could play any significant role in the reduced CTL response seen in recipients of cells from infected donors. Consequently, our results indicate a deficiency of at least one of the T-cell subsets collaborating in the generation of CTL; this deficiency is temporally related to immunosuppression in the intact mice. Analogical observations have been made concerning the T-helper cell population for humoral responses (5, 8). Although additional factors seem to be working in the intact LCMV-infected mice (4), we suggest that these T-cell

TABLE 4. Thymus-restoring capacity of bone marrow and spleen cells from infected mice after transfer to 800-R-irradiated LCMV-immunized mice or LCMV virus carriers[a]

Tested cell population (day p.i.)	Cells per donor ($\times 10^6$)		Thymus restoration of recipients				Restorative potential[b]
			Weight (mg)		No. of cells ($\times 10^6$)		
	Infected	Control	Infected	Control	Infected	Control	
Bone marrow							
(4)	3.7	7.4	26	36	52	139	19
(9)	3.6	5.6	19[c]	34	33[c]	90	24
(13)	7.0	6.9	9[c]	19	7[c]	54	13
(13)[d]	5.4	6.5	22[c]	47	38[c]	138	23
(18)	4.7	6.3	20	29	35[c]	78	33
(30)	3.8	4.7	27	30	79	85	75
Spleen cells							
(4)	104	100	9	15	5[c]	24	22
(8)	112	158	13	13	25	28	64
(13)	278	134	13	8	20	11	377
Control bone marrow[e]			21	19	55	48	

[a] Irradiated mice (800 R) were given 3×10^6 femoral bone marrow cells or 25×10^6 spleen cells. Twenty days later the weight and cellularity of the thymuses were individually determined. Unless otherwise stated, recipients were mice immunized with 10^3 times the LD_{50} of LCMV i.p. 2 to 4 months earlier.

[b] Percent response of infected donors corrected for differences in total cell number.

[c] $P < 0.05$, Wilcoxon rank test versus corresponding control.

[d] In this experiment LCMV virus carriers were used as irradiated recipients.

[e] Normal bone marrow transferred to immunized recipients given 10^3 times the LD_{50} i.p. at the time of cell transfer. Responses of uninfected recipients are shown as controls.

defects play an important role in LCMV-induced immunosuppression, and this would certainly explain why T-cell-dependant immune responses are selectively affected (5). From the experiments cited we cannot say whether the nature of the defect is an abnormal capacity of the individual T-cell or a simple numerical deficiency of immunocompetent cells. The virus-induced changes in the thymus and in the T-cell progenitor pool, however, seem to favor the latter possibility (see below).

The various mechanisms by which LCMV could affect the immunocompetent cell population have been discussed in detail elsewhere (4), and the hypothesis was advanced that an inhibition of the common myeloid and lymphoid stem cells setting in during the very first days of LCMV infection was associated with suppression of the restricted lymphoid progenitor cells and T-cell maturation leading to the later-observed deficiency of immunocompetent T-cells. However, although LCMV-induced interference with the common stem cells and with various restricted myeloid progenitor cells was observed (6), any inhibition of T-cell progenitors and the recruitment of immunocompetent T-cells were never documented. The present experiments have tested these predictions of the above-mentioned hypothesis, and the results obtained provide substantial support on two points.

First, the number of cells in the thymus decreased rapidly during the first days of the infection and was maintained at a subnormal level for several weeks. Histologically this decrease was shown to reflect marked depletion of cortical lymphocytes. Although it was previously believed that these represent a sterile differentiation pathway (21), more recent observations have shown that most emigrants derive from cortical precursors, whereas most medullary thymocytes, despite their immunocompetence, are not destined to emigrate (23, 28). Consequently, the thymic changes are compatible with the assumption that there is decreased migration out of the thymus during acute LCMV infection. Considering the rapid turnover rate of the thymic cortex as compared with the medulla and the lack of evidence for cell necrosis, the decrease in thymocyte number is most easily explained by (i) assuming an inhibition of the proliferation of thymus cells or (ii) a decrease in the input of T-cell progenitors from the bone-marrow or spleen or both, or (iii) both (i) and (ii).

Second, and most important, our results indicate that the T-cell progenitor pool of acutely infected mice is in fact reduced (Table 4). This finding supports the above explanation of the thymic changes and is consistent with the basic idea of the described working hypothesis. We cannot tell whether the reduced capacity to repopulate the thymus of irradiated recipients reflects a direct suppressing affection of the restricted T-cell progenitors, an abolished recruitment of such cells from the deficient common stem cell pool, or a combination of these mechanisms. It is essential, however, that our results rule out the possibility of T-cell recruitment being unaffected by the LCMV-induced stem cell suppression due to the existence of an unimpaired population of restricted T-cell progenitors (1). It should be added that although our results indicate a numerical deficiency of progenitor cells, it may be equally important in the intact mouse that such cells which are left over may not function; this appears to be the case for the common stem cells (7). Unfortunately, it is not possible to clarify this point by studying the process of thymus repopulation in LCMV-infected irradiated recipients since such mice would simply die from irrelevant hemopoietic failure.

Until now we have stressed the importance of cell deficiency in the T-cell lineage that seems to reflect inhibition of the differentiation and proliferation in the early stages of T-cell maturation. Although it is tempting to compare the effect of LCMV infection to adult thymectomy (13), it appears that the changes in the peripheral T-cell compartment take place too rapidly to reflect solely a "functional thymectomy" (2, 3, 19). It is conceivable, however, that in addition LCMV interferes with postthymic proliferation and differentiation (11, 23) in ways which thymectomy does not.

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