MECHANISMS OF SUPPRESSION OF CYTOTOXIC T-CELL RESPONSES IN MURINE LYMPHOCYTIC CHORIOMENINGITIS VIRUS INFECTION

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Lymphocytic choriomeningitis (LCM) virus infection of mice produces diverse disease syndromes depending upon the strain of virus, the route and dose of infection, and the age and strain of the mouse (1-5).

Historically, perhaps the most significant syndrome is the vertically transmitted (congenital) carrier state in which fetuses of infected mothers are infected in utero and exhibit chronic infection throughout their lives (1-5). These mice either do not respond immunologically to viral antigens or give chronic antibody responses which lead to immune complex-mediated glomerulonephritis (6). It was the specific unresponsiveness of such mice which led Burnet and Fenner (7) to make the fundamental postulate of the existence of a state of immunological tolerance to those antigens present in the fetal or neonatal animal during the ontogeny of the immune system. With the formulation of the "Clonal Selection Theory" (8), a more precise statement of this form of tolerance could be made, viz., that clones of immunocytes reactive to self-antigens were "forbidden" clones and were deleted during ontogeny.

So far as humoral immunity is concerned, this statement is not strictly true, since antibodies to self-antigens can be demonstrated in a variety of models (9), and there are anti-viral antibodies produced in the LCM virus carrier state (6) as mentioned above. Another important aspect of the immune response to LCM virus infection in adult mice is the generation of thymus-derived (T) cells which lyse LCM virus-infected target cells with great efficiency (10-12). Such T cells seem to be responsible for recovery from primary infection in adult animals (13, 14) and also seem to provoke another interesting syndrome in animals infected intracerebrally, a lethal acute central nervous system disease (15-18) (LCM) apparently caused by T-cell attack on LCM virus-infected cells in the choroid plexus and meninges (19). LCM virus congenital carriers do not exhibit evidence of a cytotoxic T-cell response (10, 11, 20-22). Since induction of a partially tolerant carrier state is not dependent on LCM virus antigens being present during ontogeny, but can be achieved in neonates or in adults with high doses of virus (5), a re-evaluation of the possible mechanisms involved is appropriate, particularly in view of recent indications of the active suppression by T cells of responses to many antigens, including self-components (23, 24).

In this report we describe conditions that lead to either continuation or suppression of LCM virus-specific cytotoxic T-cell responses upon transfer of responding cells to an in vitro system, or to irradiated syngeneic, recipient mice. We also describe investigations

1 Abbreviations used in this paper: Con A, concanavalin A; LCM, lymphocytic choriomeningitis; PFU, plaque-forming units; PI, post-infection; SPF, specific pathogen-free; WE3, Westminster (viscerotrophic) strain of LCM virus.
Into the suppressive effects of congenital carrier cells both in vitro and after transfer into normal recipients.

Materials and Methods

Mice and Virus Stocks. Inbred CBA/H mice of both sexes were bred in the John Curtin School and used at 6-8 wk of age. They were either from a specific pathogen-free (SPF) colony, or from a WE3 LCM virus congenital carrier colony. SPF mice were infected (i.v.) with 0.2 ml of a $10^{-4}$ dilution of a guinea pig spleen or lung stock of WE3 LCM virus containing 4,000 intracerebral LD$_{50}$ units (25) equivalent to 250 plaque-forming units (PFU). Virus stocks were stored in small portions at -70°C, and a fresh portion was used for each immunization.

In Vitro Secondary Stimulation. The procedure for stimulation of spleen cells from previously infected mice by cocultivation for varying periods with LCM virus-infected, syngeneic peritoneal cells has been described in detail elsewhere (26, 27).

In Vivo Secondary Stimulation. 1 day before transfer of the splenic responder T cells under test, recipients were given 850 rads from a $^{60}$Co source. On the day of transfer, recipients were infected i.v. with $2.5 \times 10^4$ PFU. 12 h later, spleen cells obtained from donor mice were transferred i.v. to groups of four irradiated, infected, or uninfected syngeneic recipients at $10^8$ cells per mouse. Mice were then kept for various intervals before removal of their spleens for assay of cytotoxic T-cell activity against virus-infected targets. Spleens generally yielded $5 \times 10^5 - 5 \times 10^6$ viable lymphoid cells.

Technique of $^{51}$Cr Release Assay, and Methods of Expressing Results. The assay has been described before (25). Targets were LCM virus-infected or uninfected, H-2 compatible L929 cells for all figures and tables excepting Table V. Assay incubation time was 16-18 h except with concanavalin A (Con A) blast cell targets (Table V). Results were expressed as percent specific lysis = ($\%$ $^{51}$Cr release in presence of effectors - $\%$ medium release) × 100/$\%$ water lysis. Specific lyses on infected targets only are shown for all figures and tables excepting Table V. Data presented are the means of four replicates. Standard errors of the mean were never greater than ±2% and were omitted for clarity. Statistical significance was determined by Student's t test.

Generation of Blast Cells Using Con A. Splenic lymphocytes from LCM virus congenital carriers and from normal mice were stimulated in culture with a dose of Con A previously shown to produce maximal $[^3H]$thymidine incorporation. Thus, $4.0 \times 10^7$ viable cells were cultured in large Falcon plastic flasks (Falcon Plastics, Oxnard, Calif.) in medium containing Con A (Pharmacia Fine Chemicals, Uppsala) for 5 days at 37°C. The resulting blast cells were washed and used as $^{51}$Cr-labeled targets as described for L929 cells, except that they were used at $10^6$ cells per well instead of $5 \times 10^5$, the assay was run for only 2 h at 37°C to avoid problems due to high rate of spontaneous $^{51}$Cr release, and the assay trays were lightly centrifuged (1,000 rpm for 10 min) immediately after mixing target cells and killer cells to ensure rapid contact between them.

LCM Virus Neutralizing Antiserum. Spleen cells from CBA/H mice primed 8 wk previously with LCM virus were transferred to syngeneic LCM virus carriers ($10^9$ cells per mouse). Recipients were bled 5 wk later. Serum was heat inactivated at 56°C for 30 min to destroy any infectious LCM virus present. This serum had a titer of 1:80 by neutralization of PFU in vitro.

LCM Virus Plaque Assay. This is to be described elsewhere and is similar to the assay used to titrate ectromelia virus (28).

Results

Kinetics of Virus Production in Relation to Generation of Cytotoxic T Cells in Spleens. Virus titers in spleen increased rapidly to peak on days 3-5 after virus inoculation and slowly fell thereafter (Fig. 1). Spleen cells showed peak primary cytotoxic T-cell activity on day 9; activity fell slowly and there was still significant activity (and persistent virus) by day 21 post-infection (PI) (Fig. 1). The relative cytolytic activity of day 5 PI, day 9 PI, and day 21 PI populations have been confirmed several times (data not shown).

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2 Cole, G. A., and M. B. C. Dunlop. Manuscript in preparation.
3 Dunlop, M. B. C. Manuscript in preparation.
Kinetics of Cytotoxic T-Cell Activity in Spleen Cell Populations Primed at Various Intervals with LCM Virus and then Transferred to an In Vitro System. Groups of CBA/H mice were infected i.v. with 250 PFU of WE3 LCM virus 5, 9, or 21 days before culture of their spleen cells with syngeneic, infected peritoneal "stimulator" cells. Cultures were incubated for 3, 4, 5, or 6 days before being assayed against infected L929 target cells on the same day (Fig. 2). Spleen cells from uninfected control mice did not generate cytolytic activity in culture, while day 5 PI cells did show cytolysis of infected targets which increased in activity to peak on day 5 (i.e., about that time, 10 days PI, at which these same spleen cells would be expected to reach peak effector activity if they remained in the intact infected animal). Day 9 PI spleen cells gave greater cytolytic activity than day 5 PI cells but continually declined in activity from 3 to 6 days of
culture. Day 21 PI spleen cells were very potent for the 4 days of culture assayed (Fig. 2); this is an expression of the secondary response of memory T cells (26, 27). Other results (not shown) indicated that these three primed spleen cell populations were less active after culture with uninfected, syngeneic peritoneal cells.4

**In Vivo Transfer or In Vitro Culture of Spleen Cells at Various Intervals PI with LCM virus.** Groups of CBA/H mice were infected i.v. with 250 PFU of WE3 virus at intervals, their spleen cells were harvested at various times PI, and portions of the spleen cell pools were either transferred into irradiated, infected, or uninfected syngeneic recipients and left for 4 days, or cultured with syngeneic, infected, or uninfected peritoneal cells for 4 days. Recipients' spleens and cultures were then assayed for cytotoxic activity (Table I). Day 9 PI primary cells retained cytotoxic activity against infected targets both in vivo and in vitro. They retained more potency in a deliberately infected environment than in an uninfected environment. Day 21 PI memory cells gave potent secondary responses both in vivo and in vitro, and a deliberately infected environment again gave more potent responses. It must be borne in mind, however, that virus was present in both day 9 PI and day 21 PI spleen cell populations (Fig. 1) so that some infected cells would be present, regardless of the environment into which they were transferred. This is even more relevant to the day 5 PI cells which contained the largest amounts of virus. These cells gave significant cytotoxic T-cell activity in vitro with both infected and uninfected stimulators (more with the former), but in vivo their response was largely suppressed in infected recipients, though it appeared in uninfected recipients. In several further experiments there was little evidence of the generation of cytolytic activity from day 5 PI primary cells after transfer to infected, irradiated recipients 3, 5, 6, or 8 days before assay (data not shown). Also, no activity was detected in other organ sites such as thymus, peritoneal cavity, blood, or mesenteric lymph node. Hence, this prepeak population appeared to be suppressed in the presence of excess virus in vivo.

**Suppression by Transfer to Infected, Irradiated Recipients, or Culture with Excess Infected Stimulators, and Effect of Prior Treatment with Neutralizing Antiserum.** Spleen cells from CBA/H mice infected i.v. with 250 PFU of WE3 LCM virus 5 days or 9 wk previously were transferred into infected or uninfected, irradiated recipients, or cultured with infected or uninfected stimulator cells (at a high or low stimulator:responder ratio) for 5 days before assay of recipients and cultures on the same day for cytotoxic activity against infected targets. A number of day 5 PI or week 9 PI donor mice were given 0.2 ml of LCM virus neutralizing antiserum i.v. 30 min before harvesting spleens for transfer or culture (Table II). As demonstrated in Table I, day 5 PI (pre-peak) primary cells generated little cytotoxic activity in infected, irradiated recipients compared with uninfected recipients. Moreover, cocultivation of pre-peak primary cells with an excess of infected stimulator cells (stimulator:responder ratio of 1:1) suppressed generation of cytotoxic activity compared with the cultures where a stimulator:responder ratio of 1:32 was used. In contrast, pre-peak primary cells from donors given neutralizing antiserum which presumably

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4 Dunlop, M. B. C., P. C. Doherty, R. M. Zinkernagel, and R. V. Blanden. Manuscript in preparation.
### TABLE I

**Suppression of Generation of Cytotoxic Activity of Day 5 PI Spleen Cells by Transfer to Irradiated, Heavily Infected Recipients, but not by Culture with Infected Stimulator Cells***

| Interval between priming and sacrificing donors | Status of recipient (in vivo) or stimulator cells (in vitro) | In vivo transfer | In vitro culture |
|-----------------------------------------------|----------------------------------------------------------|-----------------|-----------------|
|                                               |                                                          | E.T ratio       | Percent specific lysis on infected L929 | E.T ratio       | Percent specific lysis on infected L929 |
| Nil (uninfected)                              | Infected                                                 | 30:1 0.1        | 4:1 1.3         |
|                                               |                                                          | 10:1 0          | 1:1 0           |
|                                               | Uninfected                                               | 30:1 4.4        | 4:1 0           |
|                                               |                                                          | 10:1 1.1        | 1:1 0           |
| 5 days (pre-peak primary)                     | Infected                                                 | 30:1 7.8‡       | 4:1 24.4§       |
|                                               |                                                          | 10:1 5.7‡       | 1:1 6.3         |
|                                               | Uninfected                                               | 30:1 30.2       | 4:1 12.5        |
|                                               |                                                          | 10:1 28.8       | 1:1 4.5         |
| 9 days (peak primary)                         | Infected                                                 | 30:1 74.5§      | 4:1 85.8§       |
|                                               |                                                          | 10:1 71.0§      | 1:1 55.6§       |
|                                               | Uninfected                                               | 30:1 37.9       | 4:1 37.5        |
|                                               |                                                          | 10:1 32.1       | 1:1 9.2         |
| 21 days (memory)                              | Infected                                                 | 30:1 61.3§      | 4:1 82.0§       |
|                                               |                                                          | 10:1 54.4§      | 1:1 76.7§       |
|                                               | Uninfected                                               | 30:1 22.3       | 4:1 5.1         |
|                                               |                                                          | 10:1 14.3       | 1:1 6.9         |

* Procedures for in vivo transfer and in vitro culture given in Materials and Methods. In vivo transfer and in vitro culture were for four days. Medium release on infected targets was 23.7% in experiment 1 and 20.4% in experiment 2.

‡ Significantly less lysis (P < 0.001) than with the relevant control of uninfected irradiated recipients for same effector:target ratio on infected targets.

§ Significantly greater lysis (P < 0.001) than with the relevant controls of uninfected irradiated recipients or uninfected stimulator cells at same effector:target ratio.

reduced the amount of infectious virus, generated significantly greater cytolytic activity on in vivo transfer or in vitro culture in infected or uninfected environments (Table II).

Memory cells (9 wk PI) gave potent secondary responses in an infected environment either in vivo or in vitro at a stimulator:responder ratio of 1:32 (Table II). Uninfected environments gave little secondary response. In contrast to pre-peak (5 day PI) primary cells, the administration of neutralizing antiserum to memory cell donors did not improve expression of the secondary response in vitro at the high stimulator:responder ratio. However, memory cells were also suppressed in vitro in the presence of very large numbers of infected stimulator cells (stimulator:responder ratio of 1:1).

**Suppression of Primary Responses by Culture with Syngeneic LCM Virus**
## Table II

**Suppression of Generation of Cytotoxic Activity of Day 5 PI Spleen Cells by Transfer to Irradiated, Heavily Infected Recipients and by Culture with Excess Infected Stimulator Cells, and Abrogation of Suppression by Injection of Neutralizing Antiserum 30 min Before Obtaining Day 5 PI Cells**

| Interval between priming and sacrificing donors | Status of recipient (in vivo) or stimulator cells (in vitro) | In vivo transfer | In vitro culture (S:R:1:32)‡ | In vitro culture (S:R:1:1)‡ |
|-----------------------------------------------|-------------------------------------------------------------|-----------------|-----------------------------|-----------------------------|
|      | Neutralizing antiserum transfer E:T ratio | Percent specific lysis on infected L929 E:T ratio | Percent specific lysis on infected L929 E:T ratio | Percent specific lysis on infected L929 E:T ratio |
| 5 days | No | Infected 30:1 4.6§ 1:1 32.4 13.5§ | Uninfected 30:1 37.6 4:1 42.2 16.1 |
|       | | 10:1 5.2 1:1 12.4 |
| 5 days | Yes | Infected 30:1 19.2% 4:1 60.9% ND |
|       | | Uninfected 30:1 15.7% |
| 9 wk | No | Infected 30:1 45.9% 4:1 63.0% ND |
|       | | 10:1 22.7% |
| 9 wk | Yes | Infected 30:1 78.0 4:1 93.2 12.7§ |
|       | | 10:1 78.8 1:1 82.6 5.3§ |
|       | Uninfected 30:1 5.2 4:1 11.0 9.7 |
|       | 10:1 2.9 1:1 1.6 4.0 |

* Procedures for in vivo transfer, in vitro culture, and antiserum injections given in Materials and Methods. In vivo transfer and in vitro culture duration was 5 days. Results were the means of triplicates. SE of mean usually less than ±2% and were omitted for clarity. Medium release was 18.9% on infected targets and 17.3% on uninfected targets.

† S:R, peritoneal stimulator cell:spleen responder cell ratio.

§ Significantly less lysis (P < 0.001) than with the relevant control of uninfected irradiated recipients or uninfected stimulator cells for same effector:target ratio on infected targets.

¶ Significantly more lysis (P < 0.001) than with the relevant control without neutralizing antisera transfer for same effector:target ratio on infected targets.

**Congenital Carrier Spleen Cells.** Having determined that both primary and secondary cytotoxic T-cell responses could be suppressed in the presence of excess virus or virus-infected cells in vitro or in irradiated recipients, suppressive activity present in congenital carrier cell populations was investigated in a similar manner. CBA/H mice were infected i.v. with 250 PFU of WE3 LCM virus for 5 days, 8 days, or 9 wk before culturing with equal numbers of either syngeneic normal or carrier cells, in the presence of either uninfected or infected, syngeneic peritoneal stimulator cells. All cultures were assayed for cytotoxic activity after 5 days (Table III). Day 5 PI cells generated cytotoxic T-cell activity in cultures with normal spleen cells, but were suppressed when cultured with congenital carrier cells. Day 8 PI primary cytotoxic T cells in the presence of infected stimulators were also less active when cultured with carrier spleen cells than normal spleen cells, but in the presence of uninfected stimulators, more cytotoxic activity was generated with carrier cells than normal spleen cells. Memory cells (week 9 PI) gave strong secondary responses in all cultures.
| Component spleen populations | Stimulator status | Percent specific lysis on infected L929 |
|-----------------------------|------------------|----------------------------------------|
|                             |                  | E:T ratio 4:1  | E:T ratio 1:1 |
| Day 5 PI + normal           | Infected         | 53.1          | 12.0          |
|                             | Uninfected       | 47.8          | 10.6          |
| Day 5 PI + Congenital carrier | Infected | 19.3†         | 2.1†          |
|                             | Uninfected       | 15.1†         | 3.4           |
| Day 8 PI + normal           | Infected         | 85.1          | 29.9          |
|                             | Uninfected       | 26.9          | 5.5           |
| Day 8 PI + Congenital carrier | Infected | 55.4†         | 13.1†         |
|                             | Uninfected       | 51.1          | 11.6          |
| Week 9 PI + normal          | Infected         | 83.1          | 56.8          |
|                             | Uninfected       | 0             | 0             |
| Week 9 + Congenital carrier | Infected         | 81.6          | 71.5          |
|                             | Uninfected       | 86.0          | 69.3          |

* 1.25 × 10^7 of each component spleen population were cocultivated with infected or uninfected syngeneic stimulator cells for 5 days before assay. Stimulator:responder ratio was 1:32. Medium release was 19.6% on infected targets.
† Significantly less lysis (P < 0.001) than with the relevant control with normal spleen cell component population for same effector:target ratio on infected targets.

containing either infected stimulators or carrier spleen cells, or both, but did not respond in the absence of both. These results indicated that congenital carrier spleen cells could act as infected stimulators, presumably because of virus-induced antigens on their surface membranes (4, 5). This produced either induction of secondary responses, when the responding memory cells carried little intrinsic infection, or suppression of primary responses, where the responding cells still carried a considerable virus load, or where additional infected stimulator cells were present.

**Suppression of Primary Response In Vivo by Transfer of LCM Virus Congenital Carrier Spleen Cells (Intact or Lysed) or by an Equivalent Dose of Virus.** 2.5 × 10^7 LCM congenital carrier spleen cells were transferred to normal, syngeneic CBA/H recipients on various days before or after WE3 LCM virus infection (250 PFU i.v.) on day 0; recipients' spleens were assayed for cytotoxic T-cell activity on infected target cells 7 days later (day +7). Transfer of congenital carrier cells from 7 days before infection or as late as 3 days after infection significantly depressed the primary cytotoxic T-cell response (Table IV). Generally, suppression was greater the earlier the carrier cells were transferred. Suspensions of carrier spleen cells disrupted by water lysis and sonication, but containing infections virus, gave a similar degree of suppression to living carrier cells (Table IV). Furthermore, a dose of virus (2.5 × 10^4 FFU) equivalent to the virus content of 2.5 × 10^7 carrier spleen cells produced a degree of suppression similar to that caused by living or lysed carrier cells (Table IV). It was also
MECHANISMS OF SUPPRESSION IN LCM INFECTION

Table IV

Suppression of Primary Cytotoxic T-Cell Response In Vivo by Transfer of LCM Congenital Carrier Spleen Cells, or Lysed Carrier Cells, or an Equivalent Virus Dose*

| Day of transfer | Nature of spleen cells (or virus) transferred | Virus (2.5 x 10^7 PFU) given on day 0 | Percent specific lysis of infected L929 |
|----------------|---------------------------------------------|--------------------------------------|----------------------------------------|
|                |                                             |                                      | E:T ratio 30:1                         | E:T ratio 6:1 |
| -7             | Carrier                                     | Yes                                  | 0.9†                                   | 0† |
| -3             | Carrier                                     | Yes                                  | 11.2†                                  | 10.6† |
| -2             | Carrier                                     | Yes                                  | 20.2†                                  | 6.6† |
| -1             | Carrier                                     | Yes                                  | 10.7†                                  | 5.2† |
| -1             | Lysed carrier                               | Yes                                  | 17.3†                                  | 6.6† |
| -1             | 2.5 x 10^6 PFU of virus                     | Yes                                  | 11.3†                                  | 6.1† |
| 0              | Normal                                      | Yes                                  | 61.0                                  | 41.2 |
| 0              | Nil                                         | Yes                                  | 62.8                                  | 38.5 |
| 0              | Carrier                                     | No                                   | 26.6† §                               | 10.1‡ |
| 0              | Normal                                      | No                                   | 2.5†                                  | 2.4‡ |
| +1             | Carrier                                     | Yes                                  | 39.8†                                  | 12.2‡ |
| +2             | Carrier                                     | Yes                                  | 38.1†                                  | 22.2‡ |
| +3             | Carrier                                     | Yes                                  | 44.6†                                  | 29.8‡ |
| +6             | Carrier                                     | Yes                                  | 60.8                                  | 39.2 |

* 2.5 x 10^7 LCM congenital carrier spleen cells (intact or water lysed and sonicated) or their equivalent LCM virus content (2.5 x 10^6 PFU) were transferred i.v. at various intervals into syngeneic CBA/H recipients. Recipients were given 250 PFU intravenously on day 0, and spleens were assayed on day +7. Medium release was 20.7% on infected targets.

† Significantly less lysis (P < 0.001) than with the relevant control of normal spleen cells transferred followed by 250 PFU of WE3-LCM on day 0 for same effector:target ratio on infected targets.

§ Significantly greater lysis (P < 0.001) than with the relevant control of normal spleen cells only transferred on day 0 for same effector:target ratio on infected targets.

It is possible to produce suppression by transfer of doses of living carrier cells ranging from 5.0 x 10^7 down to as few as 5.0 x 10^6 given 1 day before infection with LCM virus (data not shown). These results suggested that infectious virus or virus-induced antigen, rather than an active suppressive process on the part of carrier cells, was responsible for suppression.

Formaldehyde-fixed carrier cells were not suppressive (data not shown) when compared with fixed normal cells in the in vitro protocol shown in Table III. Since such fixed cells display virus-induced antigenic patterns that stimulate cytotoxic T cells (26), this reinforced the view that infectious virus was the active agent of the suppression exerted by carrier cells.

It could be argued, however, that large numbers of carrier cells contaminating the cytotoxic T-cell population could act as "cold" competitors in the cytotoxicity assay, thus exerting a pseudo-suppressive effect. This was tested by adding carrier cells to cytotoxic T cells generated in their absence, immediately before mixing with labeled target cells. The number of carrier cells added was the same as that which would result if carrier cells added to the responders in the protocol shown by Table III survived in the same proportion as responders till the end of the 5-day period in vitro. No competitive effect was detectable.

Susceptibility of Virus-Infected T Cells to T-Cell-Mediated Lysis. One possible mechanism of suppression produced by excess virus is that LCM virus-
### Table V

*Susceptibility of CBA/H Con A-Stimulated Congenital Carrier Blast Cells to Lysis by Syngeneic LCM Virus-Specific Cytotoxic T Cells* Generated in a Secondary Response

| Effector spleen cells | E:T ratio | Percent specific lysis |
|-----------------------|-----------|-----------------------|
|                        |           | Congenital carrier blasts | Normal blasts |
| Normal control         | 20:1      | 0                      | 0             |
|                        | 10:1      | 0                      | 0             |
|                        | 5:1       | 0                      | 0             |
|                        | 2.5:1     | 1.9                    | 0             |
| Secondary              | 20:1      | 16.7*                  | 5.0           |
|                        | 10:1      | 11.65                  | 3.5           |
|                        | 5:1       | 11.1*                  | 3.3           |
|                        | 2.5:1     | 7.7§                   | 1.6           |

* Procedures for in vitro generation of secondary virus-specific T cells and Con A blasts are given in Materials and Methods. Medium release was 17.0% from carrier blasts and 21.7% from normal blasts over the 2-h assay.

* Significantly greater lysis (*P* < 0.001) than with the relevant control of normal spleen cells on same target at the same effector:target ratio.

§ *P* < 0.01.

Specific cytotoxic T cells are themselves infected, display virus-induced antigen patterns complementary for their own antigen-receptors, and thus either commit suicide or kill each other (29-31). To test formally whether LCM virus-infected T cells are susceptible to T-cell-mediated lysis, spleen cells from LCM virus congenital carrier or from normal mice were stimulated in vitro with the T-cell mitogen Con A (32) for 5 days and then used as labeled target cells, either infected or uninfected, respectively. LCM-infected Con A blasts were significantly lysed by syngeneic, LCM virus-specific cytotoxic T cells generated in a secondary response in vitro (Table V).

### Discussion

The evidence presented here shows that T-cell populations committed to either a primary or secondary response to LCM virus infection can be suppressed by a variety of experimental protocols which have one common feature: a high level of LCM virus, or infected cells, present in the environment of the responding T cells.

After inoculation of 250 PFC of WE3 LCM virus i.v., titers in the spleen increased for 5 days and then slowly declined. The cytotoxic T-cell response in the spleen, measured against virus-infected, *H-2* compatible targets, was detectable by day 5 PI, reached a peak on day 9 PI, and then slowly subsided. Spleen cells taken from mice before or at the peak of the response could continue the primary response either in vitro or in vivo in irradiated, syngeneic recipients, provided that correct conditions were provided. For day 5 PI cells, which themselves contained peak virus titers, it was essential not to add further virus to the system in vivo. Thus, if infected, irradiated, syngeneic recipients were used, the response was suppressed. In contrast, infected recipients gave better
MECHANISMS OF SUPPRESSION IN LCM INFECTION

maintenance then uninfected recipients of the primary response in day 9 PI cells and gave stronger induction of secondary responses in cells taken 21 days or longer after primary infection. In both of these latter cell populations, intrinsic virus levels would be 5-20-fold lower than in day 5 PI cells (Fig. 1). In vitro, maintenance of the primary response in day 5 PI cells was possible in the presence of low numbers of additional syngeneic, peritoneal, infected stimulator cells (e.g., stimulator:responder ratio of 1:32, see Tables I and II), but with high numbers of infected stimulators (e.g., 1:1, see Table II) the response was again suppressed. This was not simply due to large numbers of macrophages in the system (33), since uninfected peritoneal cells did not suppress. Furthermore, injection of LCM virus-neutralizing antiserum into 5-day PI spleen donors 30 min before removal and processing of spleens significantly improved the subsequent response in vitro, presumably by reducing the extent of spread of infection within the responding spleen cell population. Taken together, these results suggest that too much virus or too many infected cells were in some way responsible for the observed suppression of the primary response.

Spleen cells from congenital carriers were also examined for their capacity to suppress syngeneic, primary cytotoxic T-cell responses in vitro and in vivo. When added to an equal number of day 5 PI cells, carrier spleen cells significantly suppressed the continuation of the primary response in vitro, in the presence of either infected or uninfected peritoneal stimulator cells. In the same protocol, the response of day 8 PI cells was suppressed by carrier cells only in the presence of infected peritoneal cells, and there was no suppression of secondary responses (week 9 PI cells, see Table III). In fact, congenital carrier spleen cells actually stimulated responses in both day 8 PI cells and memory cells in the presence of uninfected peritoneal stimulator cells, thus indicating that they were displaying virus-induced antigenic patterns.

Virus-induced, cell surface antigens alone, however, did not seem to be the suppressive agent of carrier cells, since day 5 PI T cells continued their response in vitro in the presence of an equal number of formaldehyde-fixed carrier cells that display such antigens (26). Another potential mechanism of action of carrier cells in this type of protocol could be at the level of cytotoxic assay, i.e., pseudosuppression of the response by acting as cold competitors for the lysis of labeled targets. To do this, carrier cells would need to persist throughout the period of cocultivation in vitro, thus contaminating the cytotoxic T-cell population generated. This idea was tested by mixing carrier cells with an equal number of spleen cells taken from the peak (10 days PI) of the primary response to LCM virus in vivo; this mixture was then added to labeled target cells. The carrier cells did not compete significantly. These results thus suggested that the suppressive action of carrier cells depends upon their content of infectious virus or upon some other active process mediated only by living cells.

In vivo, transfer of doses of $5 \times 10^7$ down to $5 \times 10^5$ carrier cells to normal mice either up to 7 days before, or up to 3 days after routine infection with 250 PFU of WE3 LCM, markedly suppressed the primary cytotoxic T-cell response measured 7 days after infection, thus confirming previous results of Zinkernagel and Doherty (21). This effect could be duplicated, however, by transferring a water-lysed and sonically disrupted preparation of $2.5 \times 10^7$ carrier cells or by a dose of
2.5 x 10^4 PFU of WE3 LCM virus, the amount of virus present in 2.5 x 10^7 carrier spleen cells.

The results from all these in vitro and in vivo experiments therefore suggest a common mechanism of suppression of cytotoxic T-cell responses based upon a high level of virus (or living infected cells) being present in the environment of the responding T cells. They do not support the hypothesis that active T-cell suppression is responsible for tolerance in the cytotoxic T-cell population in LCM virus congenital carriers (1-5) or other carrier states induced by large virus doses (5).

What then could account for the observed suppression? Since LCM virus is not overtly cytopathic for lymphoid cells (4, 5), direct killing of T cells by virus seems an unlikely explanation. One possible mechanism is based upon findings that one cytotoxic T cell can kill another (29-31), provided the former has receptors complementary for antigenic patterns on the latter (30). The presence of a high level of virus in the environment of responding cytotoxic T cells specific for LCM virus-induced antigenic patterns could result in cytotoxic cells or their precursors, becoming infected and displaying antigenic patterns complementary for their own antigen receptors. This could then produce suicide of an individual cytotoxic cell, for example, in the act of endocytosis or mutual killing of adjacent cells, for example, infected, daughter, cytotoxic T cells resulting from antigen-induced mitosis of a precursor. In essence, this mechanism is the simplest implementation of the principle of "forbidden clone deletion" as suggested by Burnet (8), except that in the case of LCM virus infection it could occur in secondary lymphoid tissues during adult life as well as in the thymus, the site suggested for deletion of self-reactive clones of cytotoxic T cells during ontogeny. A direct test of the proposition is not strictly possible without methods for obtaining pure cytotoxic T-cell clones specific for LCM virus-induced antigen patterns on syngeneic cell surface membranes. However, we have presented here evidence that LCM virus-specific cytotoxic T cells can lyse infected T cells from a Con A-stimulated population (Table V). These data support the feasibility of the clonal deletion model in LCM virus carriers outlined above.

Summary

The cytotoxic T-cell response to lymphocytic choriomeningitis (LCM) virus infection was suppressed either in vitro or in vivo by addition of a high level of syngeneic virus-infected cells or syngeneic cells from congenital LCM virus carriers to the environment of the responding cells. This effect was not duplicated by formaldehyde-fixed carrier cells, nor could it be accounted for by "cold" target competition by carrier cells at the level of the cytotoxicity assay. Conversely, suppression was produced in vivo by water-lysed, ultrasonically treated carrier cell suspensions, or by a large dose of LCM virus equivalent to that contained in the carrier cells. Thus a high level of infectious virus was a common factor in all observed examples of suppression.

Based upon this, the following hypothesis, a form of "forbidden clone deletion," was proposed to account for virus-specific cytotoxic T-cell tolerance in

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5 Langman, R. E. Manuscript in preparation.
MECHANISMS OF SUPPRESSION IN LCM INFECTION

LCM virus congenital carriers, or in high dose suppression. A high level of virus in lymphoid tissues, while not cytopathic per se, may result in infection of all or most T cells; this then may lead to deletion either via "suicide" of individual, infected, cytotoxic T cells with receptors specific for virus-induced antigenic patterns on their own surface membranes, or by mutual lysis of two adjacent T cells.

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