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Secondary cytotoxic cell response to lymphocytic choriomeningitis virus

I. KINETICS OF INDUCTION *IN VITRO* AND YIELDS OF EFFECTOR CELLS

M. B. C. DUNLOP & R. V. BLANDEN *John Curtin School of Medical Research, Australian National University, Canberra, Australia*

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Summary. Secondary (memory) cell-mediated cytotoxic responses in lymphoid cells from CBA/H mice pre-primed with lymphocytic choriomeningitis virus (LCM) 5–7 weeks previously were induced by culturing these cells *in vitro* with syngeneic, infected peritoneal cells at 37° for periods of up to 5 days. Cytotoxic effectors were assayed against LCM infected, H-2 compatible target cells in a ^{51}Cr release assay. Response was greater with a higher ratio (1:10) of infected peritoneal cells:pre-primed cells than with lower ratios (e.g. 1:250). Separating responders from infected cells by a 450 mμm nucleopore membrane (coarse enough to allow passage of virus particles) still permitted induction of a secondary response whilst interposition of a 50 mμm nucleopore membrane (which apparently prevented transit of virus particles) virtually abolished the secondary response. Removal of phagocytic cells from responders prior to setting up memory cultures greatly reduced responders' capacity to be induced. Fixed, infected stimulators still induced strong secondary responses. Secondary response was maximal with spleen cells, peripheral blood lymphocytes, or pooled iliac and lumbar lymph node cells. Thymocytes responded less well, whilst mesenteric lymphoid cells and peritoneal cells gave minimal responses.

Correspondence: Dr M. B. C. Dunlop, Department of Microbiology, John Curtin School of Medical Research, Australian National University, P.O. Box 334, Canberra 2602, A.C.T., Australia.

Effector cells from memory cultures killed targets with single-hit kinetics and a rectilinear log effectors: log targets lysed relation held. Memory spleen cells developed increasing cytolytic activity from 2 to 5 days in culture. Memory-generated effectors were markedly potent by day 5, e.g. giving 70 per cent specific release at a killer:target ratio of 0·8:1. Peak DNA synthesis occurred on day 4. We conclude that memory effectors as a population differ in kinetics and potency from effectors obtained by primary viral challenge in the mouse.

INTRODUCTION

Acquisition of immunological memory to infectious agents is of crucial importance in determining host responses to subsequent infectious confrontation. As yet, memory for cell-mediated cytotoxic responses has received little attention. Assays to assess T-cell cytotoxic responses to several different viruses have been reported (Doherty and Zinkernagel, 1976; Leclerc, Gomard and Levy, 1972; Zinkernagel and Doherty, 1974; Gardner, Bowern and Blanden, 1974; Koszinowski and Thomssen, 1975). However, effector cells used in these systems have been obtained from the primary cellular response *in vivo*. Recently, Plata, Cerottini and Brunner (1975) reported that they could generate cytotoxic T cells in secondary culture from mice carrying or having

rejected a Moloney sarcoma virus (MSV)-induced tumour, by culturing these mouse lymphocytes with MSV-induced tumour cells. Different groups have also recently investigated the nature of secondary T cell-mediated *in vitro* responses to allo-antigens (Cerottini and Brunner, 1974; Häyry and Andersson, 1974) and syngeneic tumour antigens (Röllinghoff, 1974) by similar procedures.

Gardner and Blanden (1976) have recently shown that specific secondary cytotoxic T cells may be induced in lymphoid cells from mice previously infected with ectromelia virus (a cytopathic poxvirus) by culturing these lymphoid cells with other lymphoid cells expressing ectromelia virus-induced antigens, at a temperature (39°) which is nonpermissive for viral replication and cytocidal effect on the responding lymphoid cells. In view of the potential relevance of T-cell memory to recovery from repeated viral infections, and in an endeavour to analyse T-cell memory responses in a way which may lead to more general therapeutic applications, we have been prompted to examine the induction in culture of secondary responses to lymphocytic choriomeningitis (LCM) virus, a noncytopathic, budding, RNA virus of rodents and man. Memory lymphoid cells were cultured with LCM infected cells at 37° (since LCM virus is noncytocidal) and the effectors obtained from culture were assayed for cytotoxicity using a ^{51}Cr release assay. This paper examines the protocol for setup of memory cultures, and the kinetics of development *in vitro* and yields of effector cells obtained from cultures.

MATERIALS AND METHODS

Mice and virus stocks

Inbred specific pathogen-free CBA/H or BALB/c strains were used throughout. Mice were primed with the viscerotrophic (WE3) strain of LCM virus 5–7 weeks before being used as donors of memory spleen cells, except where stated otherwise. Priming dose was 0·2 ml of 10^{-5} dilution of guinea-pig spleen or liver stock (Zinkernagel and Doherty, 1974) given intravenously.

Memory cultures

Peritoneal cells, spleen cells, L929 fibroblasts and P815-X2 mastocytoma cells were tested as stimulators. Peritoneal cells were obtained by washing out the peritoneal cavities of old breeder CBA/H or BALB/c mice with ice-cold Puck's saline. Usually $0·5–1·0 \times 10^7$ peritoneal cells were obtained from each donor mouse. These cells were infected by incubation at 37° with undiluted WE3-LCM virus stock for 30 min, then washing once in culture medium. When L929 fibroblast cells were used as stimulators they were given 5000 rad from a ^{60}Co source prior to being infected *in vitro*. A line of P815-X2 mastocytoma cells continuously infected with WE3-LCM was similarly irradiated before being put in culture. Infected cells were then added to small Falcon plastic flasks (surface area 25 cm^2) containing F-15 culture medium (Gibco, New York) supplemented with 10 per cent heat-inactivated foetal calf serum, 10^{-4} M 2-mercaptoethanol and antibiotics, in an atmosphere of 10 per cent CO_2 and 7 per cent O_2 in nitrogen. There were usually $2·5 \times 10^6$ infected peritoneal cells in a volume of 10 ml of medium per flask. Following overnight culture at 37°, by which time the infected cells would be maximally expressing virus-induced antigens, spleen cells obtained from memory mice were added to the infected peritoneal cells. $2·5 \times 10^7$ Responders were always added to each plastic flask (as preliminary experiments established that this number of responders gave cytotoxic effectors of the greatest potency and high yields) and the infected cell:memory responder ratio was usually 1:10. Cultures were then incubated for usually 5 days, at which time cells were washed out, counted and used as effectors in the ^{51}Cr release assay. Cultures which contained memory cells which had responded gave yields of 50–120 per cent of the original responder number, and there were no adherent cells remaining in culture.

Technique of ^{51}Cr release assay

The ^{51}Cr release assay has been described before (Zinkernagel and Doherty, 1974). Incubation time was 16–18 h with L929 fibroblast targets and 12 h with P815–X2 mastocytoma targets. L929 fibroblasts were infected with undiluted WE3-LCM stock 20 h prior to assay whilst P815-X2 mastocytoma cells were from a continuously infected WE3-LCM line.

Results were expressed as percentage specific release

$=$(percentage ^{51}Cr release in presence of effectors $-$ percentage medium release) $\times 100$/percentage water release.

Points for Figs 2 and 4 (log:log plots) were calculated on the basis of specific release. Results were means of four replicates. Standard errors were invariably less than ±2 per cent and usually less than ±1 per cent and were omitted for clarity.

Double chamber cultures

A double chamber system separated by a nucleopore membrane similar to that used by Feldmann and Diener (1971) was employed. The upper chamber, a glass cylinder of 1 cm diameter, was sealed off by a nucleopore membrane (Millipore Company) and housed infected cells. The lower chamber, a glass cylinder of diameter 2 cm sealed at one end, contained memory responders. $1{\cdot}0 \times 10^6$ Stimulator cells alone, or $0{\cdot}2 \times 10^6$ stimulator cells plus $0{\cdot}8 \times 10^6$ responder cells were placed in the upper chamber and $4{\cdot}0 \times 10^6$ responder cells in the lower chamber. Preliminary experiments established that 50 mμm membranes were toxic when included in routine memory cultures in plastic flasks unless these membranes were boiled several times beforehand, following which cell yields were unaffected. 450 mμm Membranes did not affect cell yields from cultures.

General

Fixation of infected stimulators was performed by treatment with 2 per cent w/v formaldehyde in phosphate-buffered saline (FA) for 10 min, or 0·25 per cent w/v glutaraldehyde in phosphate-buffered saline for 15 s, followed by five medium washes. The procedures of iron carbonyl treatment to remove phagocytic cells, and lysis of heparinized blood lymphocytes have been described elsewhere (Erb and Feldmann, 1975; Doherty and Zinkernagel, 1976). Tritiated thymidine incorporation was performed as a modification of the method of Lafferty, Ryan and Misko (1974).

RESULTS

Investigation of the optimal type of infected 'stimulator' cell and the optimal 'stimulator': memory responder ratio

Preliminary experiments involved culturing memory responder CBA/H (H-2^k) spleen cells with infected or uninfected syngeneic peritoneal cells, spleen cells and irradiated L929 (H-2^k) fibroblasts; or responder BALB/c (H-2^d) spleen cells with irradiated P815-X2 (H-2^d) mastocytoma cells. Results (not shown here) indicated that infected peritoneal cells caused induction of strong secondary cytotoxic responses from memory spleen cells after a 4-day culture. Infected spleen cells were less potent stimulators whilst infected L929 fibroblasts stimulated poorly. Spleen cells, however, responded fairly strongly in the presence of infected P815-X2 mastocytoma cells. Peritoneal cells were subsequently used as stimulators in all assays.

CBA/H memory spleen cells were cultured with infected or uninfected syngeneic peritoneal cells, and at a variety of infected cell:memory responder ratios, for 2–5 days prior to assay on infected or uninfected H-2 compatible L929 fibroblasts (Fig. 1).

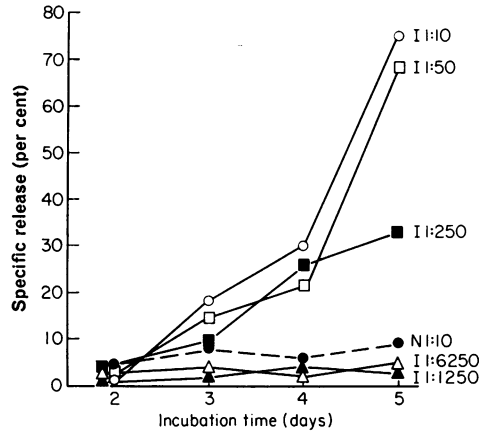

Figure 1. Effect of varying infected peritoneal cell:memory responder ratio on induction of effector activity in spleen cells from memory mice. Effectors were assayed in ^{51}Cr release assay at effector:target ratio of 0·8:1. Specific releases on infected L929 targets only are shown; medium release was 20·2 per cent. Specific releases on uninfected targets were negligible at this effector:target ratio. Standard errors were too small to be shown. Figures to the right of the curves indicate infectious status of peritoneal cells and infected peritoneal cell:memory responder ratio. I=infected; N=uninfected.

There was a continual increase in development of cytotoxic effectors from day 2 to day 5 of culture, most marked at the highest infected cell:memory responder ratio. Effectors were highly potent (about 70 per cent specific release on infected targets for an effector:target ratio of 0·8:1) on day 5 of culture for the highest infected cell:memory responder ratio (but with negligible lysis of uninfected targets at this effector:target ratio). Cultures with fewer infected peritoneal cells induced smaller cytotoxic responses. Uninfected peritoneal cells did not

Table 1. Effect of interposition of 450 mμm or 50 mμm nucleopore membrane between CBA/H stimulators and responders in a 4- and 5-day memory culture*

Type of cells in upper chamber†	Membrane pore size (mμm)	4-day Culture		5-day Culture	
		Infected L929	Uninfected L929	Infected L929	Uninfected L929
Uninfected PC	50	4·4	2·8	6·5	2·0
Infected PC		4·6	3·9	6·0	4·1
Uninfected PC	450	2·8	0	9·1	4·6
Infected PC		18·7§	7·0	23·5§	7·4
Uninfected PC+responders	50	2·2	0	8·5	3·7
Infected PC+responders		9·3	1·0	13·4	6·7
Uninfected PC+responders	450	2·0	0	6·8	4·5
Infected PC+responders		26·4§	6·8	33·1§	8·2
Uninfected PC	—‡	3·1	0	12·3	12·9
Infected PC		85·0§	7·9	88·3§	13·0

* Effectors were assayed on LCM-infected targets at a viable cell to target ratio of 10:1. Results in this and subsequent tables were expressed as percentage specific release

$$= \frac{100 \times (\text{percentage } {}^{51}\text{Cr release in presence of effectors} - \text{percentage medium release})}{\text{percentage water release}}$$

Results were means of four replicates. Standard errors were invariably less than ±2 per cent and usually less than ±1 per cent and were omitted for clarity. Medium release was 24·5 per cent on infected targets and 18·1 per cent on uninfected targets.

† $1{\cdot}0 \times 10^6$ Stimulators alone or $0{\cdot}2 \times 10^6$ stimulators plus $0{\cdot}8 \times 10^6$ spleen responders, were incubated in upper chamber. $4{\cdot}0 \times 10^6$ Responders were incubated in lower chamber. PC=peritoneal cells.

‡ No membrane present. Responders in direct contact with stimulator.

§ Significantly higher than uninfected stimulator control on infected target ($P < 0{\cdot}001$).

stimulate responders. This experiment was performed at multiple effector:target ratios and a comparison of log dose:log response curves (not shown here) confirmed these results. Increasing the stimulator:responder ratio above 1:10 in other assays (results not shown here) gave marginal or no increase in cytotoxic activity above that seen in Fig. 1, except that at the high stimulator:responder ratio of 1:1 there was strong suppression of responses (M. B. C. Dunlop, in preparation). For logistic reasons an infected cell:memory responder ratio of 1:10 was usually used in future assays.

Effect of interposition of 450 mμm or 50 mμm nucleopore membrane between infected cells and memory responders

$4{\cdot}0 \times 10^6$ Memory responders were placed in the lower chambers of the double chamber cultures, which contained $1{\cdot}0 \times 10^6$ infected cells alone or $0{\cdot}2 \times 10^6$ infected cells plus $0{\cdot}8 \times 10^6$ memory cells in the upper chambers. These double chambers were then incubated for 4 and 5 days and the effectors from the lower chambers assayed at the relatively high effector:target ratio of 10:1 (Table 1). Significant, but low, induction of effectors occurred in those cultures separated by 450 mμm membrane where infectious particles were being produced in the upper chambers. This indicates either that virus envelopes or intact virus particles can stimulate alone, or that cells capable of stimulating (after infection) are present in responder spleens. Interposition of a 50 mμm nucleopore membrane between infected cells and responders, which seems to prevent passage of LCM virus (50–120 mμm in diameter; Mannweiler and Lehmann-Grube, 1973) largely prevented induction of secondary responses in responders. Supernatants from responder chambers were inoculated intracerebrally into outbred WEHI mice; only those mice which received supernatants

Table 2. Effect of iron carbonyl treatment of memory responders prior to setting up memory cultures for 5 days*

Memory responder spleen cells	Iron carbonyl treatment	Infected stimulator peritoneal cells	Targets L929 (H-2^k)	
			Infected	Uninfected
CBA/H (H-2^k)	−	CBA/H (H-2^k)	51·1	8·7
	+		14·4†	3·7
			P815-X2 (H-2^d)	
			Infected	Uninfected
BALB/c (H-2^d)	−	BALB/c (H-2^d)	34·6	0
	+		3·2†	0·7

* Infected peritoneal cell:memory responder ratio was 1:4. Viable cell:target ratio was 2·5:1. Medium release was 18·4 per cent on infected L929 targets, 16·9 per cent on uninfected L929 targets, 27·4 per cent on infected P815 targets and 30·6 per cent on uninfected P815 targets.

† Significantly lower than control of no iron carbonyl treatment of responders, infected target ($P < 0.001$).

where infected stimulators were separated by a 450 mμm but not a 50 mμm membrane, died from viral meningitis.

Effect of iron carbonyl treatment of responders prior to setting up memory cultures

ARM-LCM primed CBA/H or BALB/c memory responders were incubated with iron carbonyl filings and magnet-treated to remove phagocytic cells prior to culturing with infected, syngeneic peritoneal cells. Effectors from these cultures were subsequently assayed on H-2 compatible (respectively L929 and P815-X2) targets. Iron carbonyl treatment, which removed 33 per cent of CBA/H cells and 20 per cent of BALB/c cells, resulted in a drastic reduction in induction of effectors in culture, when compared with untreated control cultures (Table 2), indicating an important role for iron-carbonyl adherent cells in the response.

Capacity of fixed, infected stimulators to induce secondary responses

CBA/H memory responders were cultured with uninfected, infected, formaldehyde (FA) fixed infected, or glutaraldehyde (GA) fixed infected syngeneic stimulators for 5 days prior to assay (Table 3). FA- or GA-fixed infected stimulators induced strong secondary cytotoxic responses comparable with untreated, infected stimulators, suggesting minimal requirements for *in vitro* secondary induction. Supernatants from cultures with fixed, infected stimulators did not elicit meningitis in CBA/H mice when inoculated intracerebrally.

Nature of effector dose–response activity

A dose–response assay of effectors from a 5 day assay culture was performed, using effector:target ratios varying from 2·5:1 to 0·05:1, and was plotted on log/log axes (Fig. 2). Over that part of the curve which represented less than about 30 per cent specific release, the log–log plot was rectilinear with slope of 45°, indicating single-hit killing (Miller and Dunkley, 1974; K. J. Lafferty, personal communication).

Tissue distribution of memory responders

Two separate experiments were performed with a variety of responder cell populations cultured with infected or uninfected peritoneal cell stimulators. In one experiment, spleen cells, thymus cells and mesenteric lymph node cells from LCM memory mice were used as responders, and in the other experiment, spleen cells, peripheral blood lymphocytes, peritoneal cells and pooled iliac and lumbar lymphoid cells

Table 3. Capacity of fixed, infected stimulators to induce secondary responses in CBA/H spleen cells*

Stimulator status	E:T ratio	Targets: L929 Infected	Targets: L929 Uninfected
Uninfected	0·8:1	4·5	3·7
	0·4:1	2·7	1·5
	0·2:1	0	3·3
Infected	0·8:1	71·0‡	3·1
	0·4:1	59·2	1·5
	0·2:1	34·2	0·6
FA fixed infected†	0·8:1	63·0‡	2·5
	0·4:1	38·8	1·4
	0·2:1	21·7	0
GA fixed infected†	0·8:1	50·5‡	3·5
	0·4:1	25·7	0·6
	0·2:1	13·8	0·8

* Stimulator:responder ratio was 1:15. Medium release was 28·3 per cent on infected targets and 21·0 per cent on uninfected targets.

† FA=formaldehyde-treated (2 per cent w/v for 10 min, then washed five times). GA=glutaraldehyde-treated (0·25 per cent w/v for 15 s, then washed five times).

‡ Significantly higher than release on infected targets following culture with uninfected stimulators, for same effector: target ratio ($P<0·001$).

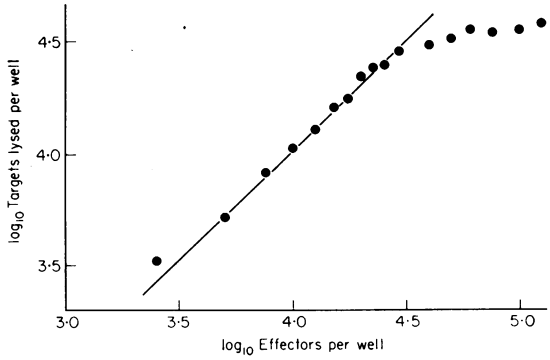

Figure 2. Plot of $\log_{10}$ targets lysed per well (=$\log_{10}$(per cent specific release × total number of targets per well)) versus $\log_{10}$ effectors/well using spleen effectors from a 5-day memory culture. Effector:target ratios ranged from 2·5:1 to 0·05:1. Infected peritoneal cell:memory responder ratio was 1:10.

Table 4. Capacity for induction of different CBA/H memory lymphoid organs *in vitro**

Responder type	Stimulator status	E:T ratio	Targets: L929 Infected	Targets: L929 Uninfected
Experiment 1†				
Spleen	Infected	10:1	74·9	38·8
		2:1	66·8	4·2
Mesenteric lymph node	Infected	10:1	25·6	11·3
		2:1	5·8	0·6
Thymus	Infected	10:1	42·4	14·6
		2:1	12·7	2·1
Experiment 2‡				
Spleen	Infected	1:1	77·4	0
		0·5:1	67·4	0·2
PBL	Infected	1:1	49·9	0
		0·5:1	31·8	0
Peritoneal cells	Infected	1:1	16·2	2·5
		0·5:1	9·1	0
Lumbar/iliac lymph nodes	Infected	1:1	75·0	0
		0·5:1	60·3	0

* Uninfected stimulators did not induce a significant response and were omitted for brevity. Stimulator:responder ratio was 1:10. Medium release was 24·3 per cent on infected targets and 20·5 per cent on uninfected targets in experiment 1, and 19·6 per cent on infected targets and 15·1 per cent on uninfected targets in experiment 2.

† Culture for experiment 1 was for 4 days, using memory mice primed 26 days previously.

‡ Culture for experiment 2 was for 5 days using memory mice primed 40 days previously. PBL=peripheral blood lymphocytes.

were the responders. Results are shown in Table 4. Spleen cells, peripheral blood lymphocytes and pooled iliac/lumbar lymph node cells responded strongly, thymocytes responded less well, and peritoneal cells and mesenteric lymph node cells responded poorly (best seen at the 2:1 effector: target ratio for experiment 1, and 1:1 effector: target ratio for experiment 2). For logistic reasons, spleen cells were used as responders in later assays.

Kinetics of development and yields of memory effectors in culture

CBA/H memory spleen cells were cultured in the presence of infected or uninfected peritoneal cells for periods up to 5 days, such that the cytotoxic

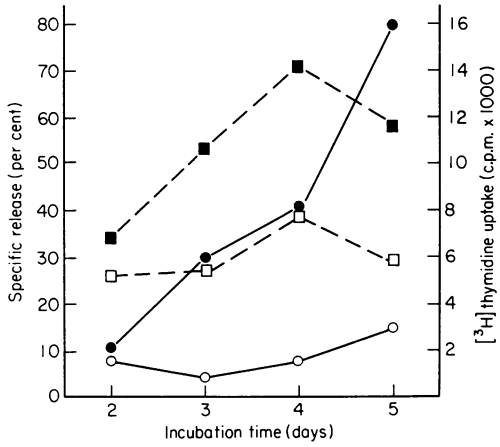

Figure 3. Time-course of development of cytotoxicity (——) and [^{3}H]thymidine uptake (– – –) for memory spleen cells cultured with infected (● and ■) or uninfected (○ and □) peritoneal cells. Peritoneal cell:memory responder ratio was 1:4. Effector:target ratio was 4:1. Specific releases on infected L929 targets only are shown; medium release was 25·9 per cent. Specific releases on uninfected targets were negligible at this effector:target ratio. Results were very similar at lower effector:target ratios.

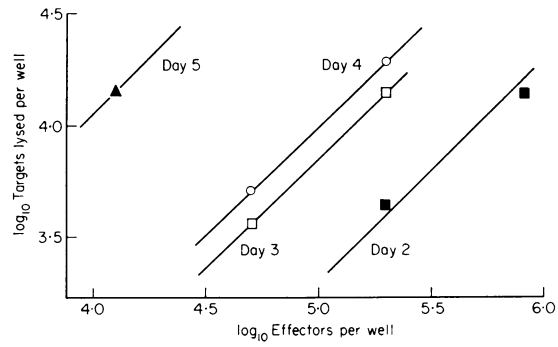

Figure 4. Plots of $\log_{10}$ targets lysed per well ($=\log_{10}$ (percentage specific release × total number of targets per well)) versus $\log_{10}$ effectors per well for memory cells obtained from 2 to 5 days in culture with infected peritoneal cells. Infected peritoneal cell:memory responder ratio was 1:4. Results were from same experiment depicted in Fig. 3.

assay was performed on the one day. [^{3}H]thymidine uptake was determined on cells from the same cultures (Fig. 3). There was a steady increase in cytotoxic effector activity from days 2–5 of culture with infected peritoneal cells. Culturing effectors for periods longer than 5 days in other assays (not shown here) showed a plateau or gradual diminution in cytotoxic activity for days 6 and 7 when memory responders came from mice pre-primed more than 3 weeks previously. Peak thymidine uptake occurred on day 4. The cytotoxic assay was performed at several effector:target ratios and a log–log plot of effector activity is seen in Fig. 4. An assumption is made (on the basis of data given in Fig. 3) that cytotoxic effectors from LCM memory cultures obey the same mathematical theory developed for MLC-induced effectors (Miller and Dunkley, 1974) and that a straight line with 45° slope drawn through the point or points which represent less than about 30 per cent specific release is a valid line.

Memory cultures became increasingly more potent with duration of culture, that is, the lines shifted upwards and to the left (Fig. 4). By determining the value of the intercept of each straight line with the abscissa it is possible to calculate effector unit activity, defined here as the reciprocal of the anti-logarithm of the intercept value (a measure of the incidence of effector cells in the memory population, and/or their individual potency; Cerottini and Brunner, 1974). Table 5 lists yields of effector cells from memory cultures. Hence, the number of effector units per 10^6 cells increased greatly and there was an associated doubling of yield from cultures from day 2 to day 5. (The low yield on day 4 is probably due to experimental conditions.)

DISCUSSION

We have described a culture system which permits induction of secondary responses in lymphoid cells previously educated to LCM virus, by incubating memory lymphoid cells with other infected lymphoid cells. It seems most unlikely that the infected cells (usually peritoneal cells) were the effectors in the ^{51}Cr release assay since naive responders cannot be induced to give specific killing of infected targets, following culture with infected peritoneal cells (results not shown here); as well, peritoneal cells were poor memory responders. LCM infected peritoneal cells were the best 'stimulators', followed by spleen cells and P815-X2 mastocytoma cells, whilst L929 fibroblasts 'stimulated' induction poorly. This property was true for ectromelia virus also (I. Gardner, Ph.D. thesis, A.N.U. Canberra) and may be related to the high proportion of macrophages in the peritoneal cell population. Macrophages may be highly efficient at presenting

Table 5. Yields of effector units from memory cultures*

Days of culture	Abscissa intercept	Effector units† per 10^6 cells ($\times 10^3$)	Yield from‡ cultures ($\times 10^6$)	Effector units per culture ($\times 10^4$)
2	1·7	22	12·6	27
3	1·2	71	12·0	84
4	1·0	100	5·4	54
5	0·05	890	23·7	2100

* Results were for same experiment depicted in Fig. 4.

† Effector units were defined here as the reciprocal of the antilogarithm of the intercept value.

‡ Cultures initially contained 25×10^6 memory spleen cells.

virus-induced antigenic patterns, by analogy with the ability of guinea-pig macrophages to present purified protein derivative (PPD) to elicit proliferative responses in PPD-immune lymph node cells (Waldron, Horn and Rosenthal, 1974) or the ability of mouse macrophage-bound hapten–carrier conjugates to elicit secondary anti-hapten antibody responses (Katz and Unanue, 1973). The fact that P815-X2 mastocytoma cells can act as 'stimulators' is possibly attributable to these cells also being of lympho-myeloid character. A related fact may be the ability of P815 cells to act as stimulators if present in low numbers in MLC (K. J. Lafferty, unpublished observations). The opposite applies for L929 fibroblasts, which stimulate poorly, if at all, in memory cultures and in mixed lymphocyte culture (MLC) (Wagner and Wyss, 1973).

Induction of secondary responses was greater with higher infected peritoneal cell:memory responder ratios, probably because more cells were presenting antigen allowing induction of more precursors (memory cells). Increasing the peritoneal cell:responder ratio higher than 1:10 only marginally increased cytotoxic activity of responders, presumably because responders were already nearly maximally stimulated. Cultures were suppressed when a high stimulator:responder ratio of 1:1 was used, possibly because macrophages in the stimulator population were nonspecifically activated following incubation with guinea-pig spleen virus stock, and in sufficient numbers to phagocytose responder cells; culturing responders with spleen cells from LCM virus carrier mice did not suppress secondary responses, even though carrier spleen cells readily suppressed primary induction on *in vivo* transfer (M. B. C. Dunlop, manuscript in preparation and unpublished results).

The nature of the true stimulator cell in our cultures is as yet unknown since the virus can, of course, be liberated in culture and can infect cells, including macrophages, in the responder population. Agents potentially capable of inducing secondary responses include mitogenic factor(s) secreted by responder cells, virus envelopes, viral proteins and intact virus particles. Provision of a source of virus (infected stimulator cells separated from responders by a 450 mμm membrane, coarse enough to allow passage of LCM virus but not cells) was able to induce weak secondary responses *in vitro* (in comparison with the control of stimulators in direct contact with responders) indicating that free virus particles in culture are not as important as direct cell-to-cell contact for induction. Mitogenic factor(s), virus envelopes and viral proteins do not appear to be important in activation of responders since interposition of a 50 mμm membrane (which allowed their passage whilst apparently preventing virus particle transit) virtually abolished secondary induction.

Removal of iron-carbonyl adherent and/or phagocytic cells (probably phagocytic mononuclear cells) from memory responders greatly reduced responders' ability to be induced, so macrophages in the responder population probably have a role in secondary induction or proliferation of memory cells. Why they should be required and whether they have a specific or nonspecific role has yet to be determined.

Formaldehyde- or glutaraldehyde-fixed infected stimulators induced strong secondary cytotoxic responses. This is analogous to the finding by Dennert and Lennox (1974) that glutaraldehyde-fixed P815-X2 mastocytoma cells could stimulate secondary *in vitro* cytotoxic responses in spleen cells

from P815-X2-primed C57Bl/6 mice. Similarly, Engers, Thomas, Cerottini and Brunner (1975) showed ready *in vitro* secondary cytotoxic cell induction using particulate alloantigens. Such assays illustrate the minimal requirements for secondary induction. Perhaps cytotoxic precursors are more efficiently re-induced or more plentiful following priming, since memory responders stimulated with the T-cell mitogen Concanavalin A show cytotoxic activity towards virus-infected targets (Cole and Dunlop, in preparation).

The memory effector cells obeyed the mathematical relationships stated by Miller and Dunkley (1974) for MLC effectors in that they gave straight line log dose:log response relationships and showed single hit killing. This, and other evidence in the accompanying paper (Dunlop, Doherty, Zinkernagel and Blanden, 1976) suggests that effector cells are T cells.

The capacity for induction of secondary responses resided to different degrees in different lymphoid organs—it was greatest in spleen, peripheral blood lymphocytes and pooled iliac/lumbar lymphoid cells, less in thymus cells and least in mesenteric lymphoid cells and peritoneal cells. Almost certainly memory cells to LCM virus are present in a recirculating pool involving spleen, blood and systemic lymph nodes. Paradoxically, precursors of memory effectors have been shown to reside in peritoneal cells but to be sparse in pooled lumbar/iliac/popliteal lymphoid cells in the ectromelia system (Gardner and Blanden, 1976). This may reflect different sites of viral multiplication following intravenous injection of different viruses, different recirculation patterns of committed memory cells, or the fact that LCM-primed mice were used 40 days and ectromelia mice 16 days post-immunization.

Cytotoxic potency and yield of effector units increased steadily from days 2–4 of culture, but increased dramatically between days 4 and 5. In other experiments, cytotoxic activity remained at a plateau or decreased slightly from day 5 to day 7. These results are consistent with peak thymidine uptake (and therefore proliferation) occurring on day 4, although it is not yet certain what proportion of the proliferating cells are effectors. The kinetics of development of cytotoxic effectors followed closely the kinetics of ectromelia memory cultures (Gardner and Blanden, 1976) and primary MLC (Cerottini & Brunner, 1974). Memory MLC, however, proliferate a little more rapidly and intensely (Cerottini & Brunner, 1974). *In vitro* secondary responses in the MSV system using MSV tumour cells as stimulators appear sooner and reach peak cytotoxic levels about 10-fold higher than primary *in vitro* responses (Plata *et al.*, 1975). Primary induction in culture in both the LCM and ectromelia systems has not as yet been achieved. Development of primary cytolytic activity in the specific pathogen-free mouse following LCM virus challenge is slower and less potent when compared with that of secondary induction with LCM; in the primary response of specific pathogen-free CBA/H mice, peak activity occurs at 7–9 days and effector unit concentration in the spleen is usually 6- to 7-fold less than in secondary cultures at 5 days (M. B. C. Dunlop, unpublished data).

It is apparent that secondary induction of LCM memory cells from certain lymphoid organs leads to a very potent effector population, and that this induction requires (only?) a source of virus or virus-induced antigen and a carbonyl iron-adherent accessory cell. There is probably an increased number or avidity of precursor cells within pre-primed lymphoid organs in comparison with virgin lymphoid tissues, but their precise relationship to the effectors from cultures is still unknown. In the accompanying paper the nature and specificity of the effector cells from memory cultures are ascertained. Further experiments will dwell on the development of memory in lymphoid cells following LCM priming, the nature of regulatory mechanisms evident soon after viral challenge, and the role of memory effectors in assisting recovery from LCM infection.

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