PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 151, 348-350 (1976)

Induction of Secondary Cytotoxic T-Lymphocytes *in Vitro* Does Not Require Cell Proliferation[1] (39207)

MARTIN RÖLLINGHOFF AND HERMANN WAGNER

(Introduced by Z. Ovary)

Institut für medizinische Mikrobiologie der Universität Mainz, D 65 Mainz, Hochhaus Augustusplatz, Germany

Murine T-lymphocytes respond *in vitro* to antigens coded by the major histocompatibility complex of the mouse by cell proliferation, which finally yields in the generation of cytotoxic T-lymphocytes (CTL). The peak of the proliferative response occurs on Day 3, whereas the peak of the cytotoxic response takes place on Days 5-6 (1-3). Evidence exists that by further cultivation CTL revert to small-sized lymphocytes. During this time period the cells lose their cytotoxic activity (4-5). Because such cells respond upon antigenic reexposure with an accelerated and enhanced generation of secondary CTL (5-6), in the following these cells will be designated as "nonlytic" secondary T-lymphocytes (NLST). It is known that in a primary cytotoxic allograft response no cytotoxic function will be generated unless the antigen reactive T-cells are able to divide (1-3). Thus apart from antigenic stimulation cell division appears to a prerequisite for the induction of cytotoxic functions within T-lymphocytes. This report deals with the observation that under certain conditions, cytotoxic functions can also be induced in mouse T-lymphocytes in the absence of cell proliferation.

Materials and methods. CBA/J ($H\text{-}2^k$) anti BALB/c ($H\text{-}2^d$) CTL were generated in a primary mixed lymphocyte culture (MLC) (2) in Linbro multiculture trays (TC-24) by cocultivating 4×10^6 CBA splenic responder cells with 1×10^6 irradiated (3000 R) BALB/c splenic stimulator cells in each culture well, as described in detail elsewhere (7); after 4 days of cultivation the cultures were harvested, pooled, and the cytotoxic activity of the CBA anti BALB/c CTL generated was tested in a 3-hr cytotoxicity assay (8). ^{51}Cr-labeled P815 mastocytoma cells ($H\text{-}2^d$) were used as target cells. The CTL generated were able to lyse 70-85% of the target cells, when a ratio of two attacker cells to one target cell was used. Since at Day 4 the CTL were found to be large-sized blast cells (1-3, 9), in a second step we were able to use the velocity sedimentation technique at 1 g (7, 10) in order to enrich for CBA anti BALB/c CTL. In a third step the purified blast cells, enriched for anti $H\text{-}2^d$ CTL were cultured together with irradiated syngeneic CBA spleen cells for a period of 10 days. During this time period the primary CTL reverted to "nonlytic" secondary T-cells.

Results. On the one hand, NLST are derived from the blast cells generated in a primary MLC; on the other hand, NLST are enriched for antigen-reactive T-cells against a given alloantigen. Therefore these cells were used to ask the question posed above, namely whether or not the generation of cytotoxic activity must be preceded by the division of the antigen-reactive responder cells.

NLST were prepared as outlined above and part of the cells were treated with mitomycin C (at a final concentration of 50 μg/ml for 30 min at 37°) (11). Subsequently untreated and mitomycin-treated NLST (1×10^6) were cultured either with syngeneic-irradiated (3000 R) CBA-spleen cells (3×10^6) or with the same allogeneic stimulator cells against which the primary induction of CTL was performed. After various time intervals the cultures were harvested and the specific cytotoxic activity generated was tested with ^{51}Cr-labeled P815 target cells. The results obtained (Table I) show that the antigen stimulus exerted by the allogeneic stimulator cells triggered in NLST cytotoxic function within 16-32 hr. Maximal cytotoxic activity was induced as early as 48-64 hr

[1] Supported by the SFB 107 (Mainz) of the Deutsche Forschungsgemeinschaft.

TABLE I. INDUCTION OF CYTOTOXIC ACTIVITY IN MITOMYCIN C PRETREATED NONLYTIC SECONDARY T-LYMPHOCYTES (NLST-CELLS).[a]

Cocultivation of	Time period of cocultivation (hr)	Specific lysis of ^{51}Cr-labeled (%) P815 target cells, Ratio of lymphocyte to target cells 3:1	P815 target cells, 1:1	EL 4 target cells, 3:1
(1) CBA anti BALB/c NLST plus syngeneic-irradiated CBA spleen cells	0	3	−1	2
	16	2	2	1
	32	5	3	−3
	48	2	−1	2
	64	4	2	2
(2) CBA anti BALB/c NLST plus irradiated BALB/c stimulator cells	0	3	−1	2
	16	20	14	3
	32	43	29	2
	48	82	61	4
	64	92	68	−0
(3) CBA anti BALB/c NLST-Mito plus irradiated BALB/c stimulator cells	0	−1	2	1
	16	22	15	2
	32	38	27	3
	48	52	37	−1
	64	43	31	2

[a] Nonlytic secondary CBA anti BALB/c T-lymphocytes (NLST) were obtained by a three-step procedure: (a) In order to generate primary cytotoxic T-lymphocytes (CTL), cultures of CBA (H-2^k) splenic responder cells (4×10^6) and irradiated (3000 R) splenic BALB/c (H-2^d) stimulator cells (1×10^6) were set up and cultured for 4 days in Dulbecco's modified Eagle's medium (Gibco) containing 100 units/ml of penicillin and 100 μg/ml of streptomycin, supplemented with 5% heat-inactivated fetal calf serum and 2 mercaptoethanol at a final concentration of 2×10^{-5} *M* (7). (b) The CTL generated were enriched for blast-cells by separation according to their size, using the velocity sedimentation technique at 1 g (7, 10). (c) Pooled blastoid CTL of a sedimentation rate of 5–7 mm/hr (specific cytotoxic activity against ^{51} Cr-labeled P815 of 83% within 3 hr at a ratio of one lymphocyte to one target cell) was cultured together with irradiated (3000 R) syngeneic CBA spleen cells at a ratio of 1:1 (2×10^6 viable cells each) for 10 days; subsequently the remaining viable cells were tested for specific cytotoxic activity (3% lysis of P815 target cells at a ratio of 2 lymphocyte to 1 target cell) and were taken as source of NLST. NLST cells (1×10^6) were used as responder cells either untreated or pretreated with mitomycin C (30 min at a final concentration of 50 μg/ml) (abbreviated by the suffix mito) and cultured together with irradiated (3000 R) syngeneic CBA or allogeneic BALB/c splenic stimulator cells (3×10^6). After various periods of time the cytotoxic activity generated was tested in a 3 hr cytotoxicity assay against ^{51} Cr-labeled P815 (H-2^d) and EL 4 (H-2^b) target cells (8).

after antigenic exposure. These findings are in full agreement with earlier work (4, 5, 12). Surprisingly NLST treated with mitomycin C also generated antigen specific cytotoxic function. In fact, within the first 32 hr the cytotoxic activity induced was almost similar in magnitude as observed in NLST that had not been treated with mitomycin C. The effectiveness of the mitomycin C treatment was controlled by the use of the [^{3}H]thymidine uptake technique (13). The results in Table II strongly suggest that NLST exposed to an allogeneic stimulus are driven into cell division as indicated by the augmented isotope uptake; however, mitomycin C-treated NLST are not.

Discussion. So far the results are compatible with the view that the induction of cytotoxic function is a phenomenon which can take place in the absence of cell division. However, the results included in Table I also suggest that the magnitude of inducible cytotoxic activity in a given population of NLST is increased provided the cells are able to divide. Thus 64 hr after antigenic reexposure the cytotoxic activity induced in normal NLST was almost twice as high, compared to mitomycin C-treated cells. Therefore the enhanced cytotoxicity observed in normal NLST 64 hr after antigenic reexposure appears to be the result of an increase in numbers of CTL by cell division. Two obvious explanations may be offered for the results presented. On the one hand, it is conceivable that we are dealing with a quantitative phenomenon, i.e., the finding of induction of cytotoxic function in the absence of cell division may be explained by the high number of antigen-reactive cells present in the population of the responder cells used. On the other hand, the responder cells used here were "nonlytic" secondary T-lymphocytes; that is, they had already encountered alloantigen. Moreover, unlike unprimed T-lymphocytes, NLST can be provoked by mere serologically defined histocompatibility antigens (i.e., classical H-2 antigens) to generate an enhanced and ac-

TABLE II. [^{3}H]THYMIDINE UPTAKE OF "NONLYTIC" SECONDARY T-CELLS (NLST) FOLLOWING EXPOSURE WITH ALLOANTIGEN.[a]

Mixed cell culture			[^{3}H]thymidine uptake cpm ± SE			
			16 hr	32 hr	48 hr	64 hr
CBA anti BABL/c	NLST	plus syngeneic-irradiated CBA spleen cells	1420 ± 270	1280 ± 130	980 ± 190	910 ± 210
	NLST	plus irradiated BALB/c stimulator cells	4280 ± 520	16340 ± 1310	11710 ± 1290	9200 ± 850
	NLST-Mito	plus irradiated BALB/c stimulator cells	1640 ± 260	1110 ± 240	1030 ± 190	390 ± 70
	NLST-Mito	irradiated BALB/c stimulator cells	1380 ± 150	990 ± 130	940 ± 80	260 ± 20
			200	60	30	20

[a] CBA anti BALB/c NLST were generated as described in Table 1. NLST (1×10^6) either untreated or pretreated with mitomycin C (50 μg/ml, for 30 min at 37°) were cultured together with irradiated splenic stimulator cells (3×10^6). After various periods of time the cultures were pulsed with [^{3}H]thymidine at a final concentration of 1 μCi/ml for 7 hr and tested for thymidine uptake.

celerated cytotoxic response (14, 15). Therefore, we propose that the capacity of T-lymphocytes to generate cytotoxic function in the absence of cell division reflects a state of activation restricted to secondary T-lymphocytes.

Summary. Using a mouse *in vitro* allograft model, evidence has been obtained that, in contrast to the accepted view, the generation of cytotoxic effector function in T-lymphocytes does not necessarily require cell division.

1. Häyry, P., Andersson, L. C., Nordling, S., and Virolainen, M., Transplant. Rev. **12,** 91 (1972).
2. Wagner, H., Röllinghoff, M., and Nossal, G. J. V., Transplant. Rev. **17,** 3 (1973).
3. Cerottini, J. C., and Brunner, K. T., Adv. Immunol. **18,** 67 (1974).
4. Andersson, L. C., and Häyry, P., Scand. J. Immunol. **3,** 461 (1974).
5. MacDonald, H. R., Engers, H . D., Cerottini, J. C., and Brunner, K. T., J. Exp. Med. **140,** 718 (1974).
6. Andersson, L. C., and Häyry, P., Eur. J. Immunol. **3,** 595 (1973).
7. Röllinghoff, M., Pfizenmaier, K., Trostmann, H., and Wagner, H., Eur. J. Immunol., **5,** 560 (1975).
8. Wagner, H., and Röllinghoff, M., Eur. J. Immunol. **4,** 745 (1974).
9. MacDonald, H. R., Phillips, R. A., and Miller, R. A., J. Immunol. **111,** 575 (1973).
10. Miller, R. G., and Phillips R. A., J. Cell. Physiol. **73,** 191 (1969).
11. Bach, F. H., and Voynon, N. K., Science **153,** 545 (1966).
12. MacDonald, R. H ., Cerottini, J. C., and Brunner, K. T., J. Exp. Med. **140,** 1511 (1974).
13. Wilson, D. B., J. Exp. Med. **126,** 625 (1967).
14. Wagner, H., and Röllinghoff, M., Transplant. Proc. **7,** 267 (1975).
15. Röllinghoff, M., and Wagner, H., Eur. J. Immunol., **5,** 875 (1975).

Received September 2, 1975. P.S.E.B.M. 1976, Vol. 151.