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Cell-mediated Allograft Responses *in vitro*

VI. STUDIES ON MACROPHAGE-MEDIATED CYTOTOXICITY

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Summary. Normal murine peritoneal macrophages were rendered cytotoxic against ^{51}Cr-labelled allogeneic and syngeneic target cells by incubation with supernatant of selected cell cultures.

'Active' culture supernatant was produced both by specifically sensitized cytotoxic T lymphocytes as well as by mitogen-stimulated T cells, but not by mitogen-stimulated B cells. The *in vitro* induced macrophage-mediated cytotoxicity was found to be non-specific in the sense that ^{51}Cr-labelled target cells of different H-2 haplotype were lysed equally well.

INTRODUCTION

There is general agreement that the capacity to mediate cytotoxic effector functions in cell-mediated immunity is a feature of at least three different cell types. It has been established, that T lymphocytes activated both *in vivo* (see review by Cerottini and Brunner, 1974) and *in vitro* (Feldmann, Cohen and Weckerle, 1972; Häyry, Anderson, Nordling and Virolainen, 1972; Berke and Amos, 1973; Wagner, Röllinghoff and Nossal, 1973) against a variety of cell surface antigens are capable of lysing autonomously target cells carrying the relevant antigen (Golstein, Wigzell, Blomgren and Svedmyr, 1972). Specific cytotoxicity has also been reported in the case of 'antibody-dependent cytolysis' (MacLennan, Loewi and Howard, 1969; Perlmann and Holm, 1969; Schirrmacher, Rubin, Pross and Wigzell, 1974) a phenomenon which takes place provided K cells are in close contact with antibody-coated target cells.

Cytotoxicity also appears to be a characteristic of 'activated' macrophages (Evans and Alexander, 1972a; Hibbs, Lambert and Remington, 1972; Lohmann-Matthes, Schipper and Fischer, 1972; Keller, 1973; Melsom and Seljelid, 1974). However, dependent on the assay system used, there is equivocal evidence for either specific (Evans and Alexander, 1972a, b; Lohmann-Matthes, Schipper and Fischer, 1972; Lohmann-Matthes, Ziegler and Fischer, 1973) or non-specific (Dimitriu, Dy, Thomson and Hamburger, 1975; Hibbs *et al.*, 1972; Melsom and Seljelid, 1973) macrophage-mediated cytotoxicity.

In order to investigate the cytotoxic function of 'activated' macrophages, we used the macrophage monolayer technique described by Lohmann-Matthes and Fischer (1973). The results obtained suggest that factor(s) released by specifically stimulated T lymphocytes are capable of activating macrophages to become non-specific cytotoxic effector cells.

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MATERIALS AND METHODS

Mice

C57Bl/6, DBA/2, BALB/c and CBA/J mice, purchased from Gl. Bomholtgard, Ry, Denmark, were used throughout. The mice were aged 6–8 weeks.

Target cells

Besides the tumour cell lines P815 (H-2^d) and EL4 (H-2^b), concanavalin A (Con A) transformed lymphoid cell blast were used. Such cells were obtained by pooling replicate cultures containing 5×10^6 normal splenic lymphocytes which had been cultured for 3 days in medium containing 2·5 μg/ml Con A (Pharmacia, Sweden). Target cells were labelled by incubating 5×10^6 cells in 1 ml of culture medium containing 100 μCi of ^{51}Cr (sodium chromate) (Radiochemical Centre Amersham, Buchler, Frankfurt, Germany).

Peritoneal macrophages

Three different methods were used.

(a) Following the i.p. injection of 2–3 ml of sterile thioglycollate medium, 'induced' peritoneal cells (PC) were collected 4–5 days later by washing out the peritoneal cavity with sterile Dulbecco's modified Eagle's medium (DMEM) (catalogue number F-16, Grand Island Biological Company). In selected experiments, the PC were enriched and separated from the bulk of lymphocytes on the basis of their size by using the velocity sedimentation gradient technique at 1 ***g*** (Miller and Phillips, 1969). An example of such a separation experiment is given in Fig. 1.

(b) PC were collected directly by washing the peritoneal cavity with fresh medium ('non-induced PC'). The PC were either enriched by velocity sedimentation or used directly.

(c) PC (either non-induced or induced) or the macrophage cell pool obtained after velocity sedimentation (see Fig. 1) were incubated (2–3 hours) on surface-treated plastic dishes. After extensive washings the adherent cells were recovered by trypsinization in the presence of EDTA.

Macrophage cultures

Macrophages ($4–5 \times 10^5$) suspended in 2 ml of DMEM supplemented with 10 per cent foetal calf serum (FCS) were seeded into Linbro dishes (16 mm diameter) (Linbro, FB-24 Fc Linbro Chemicals, New Haven, Connecticut) resulting in a cell density of about 2000 cells per mm^2 surface area. This cell density was optimal to obtain a confluent cell monolayer within 3–4 hours of incubation.

In vitro *induction of specifically sensitized cytotoxic T lymphocytes (CTL)*

The technique used for sensitization of murine T cells against H-2 alloantigens has been described in detail (Wagner, 1973b).

Production of 'active' supernatants

Two different methods were used.

(a) Specifically sensitized CTL (20×10^6) were incubated in a volume of 4 ml together with $0{\cdot}2 \times 10^6$ allogeneic tumour-target cells (ratio 100 to 1) in 35-mm petri dishes (Fa. Greiner, Nürtingen, Germany). After 24 hours of cocultivation the cells were harvested and

centrifuged. The supernatant was collected and filtered through 0·22 μm Millipore filters.

(b) Splenic lymphocytes (4×10^6) were cultured over a period of 3 days in Linbro-plates in the presence of either 5 μg/ml of lipopolysaccharide (LPS) (Difco Laboratories, Detroit, USA) or Con A (2·5 μg/ml) or phytohaemagglutinin-P (PHA) (Grand Island Biological Company, USA) at a final dilution 1:500 of the stock solution. The cells were collected, washed twice and recultured in DMEM for additional 24 hours. Thereafter the supernatant was collected and filtered through 0·22 μm Millipore filters. Supernatants were also collected from cultures containing embryonal fibroblasts (second to fourth generation) and from suspension cultures of P815 and EL4 tumour cells.

In vitro *activation of macrophages*

Macrophage monolayer cultures (derived from either induced or non-induced PC) were incubated with supernatant derived from different cell cultures (see above) for various lengths of time (0–24 hours). The macrophage monolayers were washed twice and thereafter tested for cytotoxicity.

Cytotoxicity assay

(a) The technique for testing cytotoxicity mediated by *in vitro* sensitized T cells has been reported (Wagner and Röllinghoff, 1974).

(b) The macrophage cytotoxicity assay as described by Lohmann-Matthes and Fischer (1973) was performed by incubating (for 18 hours) either normal or 'activated' macrophage monolayers (5×10^5 cells) together with a graded number of ^{51}Cr-labelled EL4 or P815 target cells in a final volume of 2 ml. Aliquots of the supernatants (1 ml) were carefully harvested and counted in a Packard Autogamma Tri-Carb scintillation spectrometer. In order to obtain the total counts, the sediment as well as the supernatant of P815 and EL4 control cultures were counted. Maximum releasable radioactivity was obtained by freezing and thawing (three times) the target cells. Results are expressed as percentage of lysis according to the formula: percentage lysis = [(^{51}Cr release by activated cells − ^{51}Cr release by control cells)/(maximum ^{51}Cr release − ^{51}Cr release by control cells)] × 100.

All determinations were performed in triplicates. Standard deviations (s.d.) were calculated in a Wang computer.

RESULTS

ACTIVATION OF MACROPHAGES BY PRODUCTS OF CYTOTOXIC T LYMPHOCYTES (CTL)

First, we investigated whether 'factors' produced in cultures containing highly active CTL would render normal macrophages specifically cytotoxic. The rationale of the experimental approach was first to activate *in vitro* murine T responder cells against allogeneic stimulator cells. In order to allow the putative 'factors' to be produced, we intended to incubate the CTL generated together with the respective target cells. Next, induced PC, enriched for macrophages by velocity sedimentation at 1 ***g*** (see Fig. 1) were to be incubated together with the supernatant obtained from CTL ('activation' period). Finally the 'activated' macrophages were to be tested for antigen-specific or non-specific cytotoxicity.

The results given in Table 1 demonstrate that *in vitro* sensitization of murine T cells against mitomycin C-treated allogeneic stimulator cells yielded specific CTL. That CTL

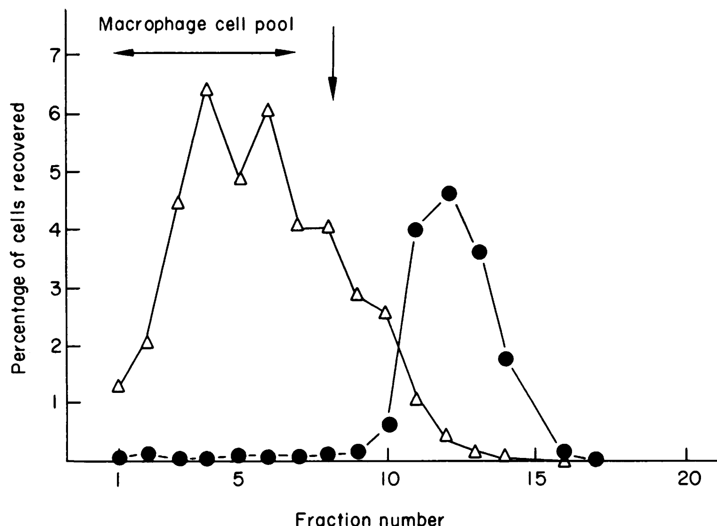

FIG. 1. Separation of PC by velocity sedimentation at 1g : 70 x 10^6 thioglycollate-induced PC suspended in 3 per cent (vol/vol) horse serum in PBS were applied to a gradient of 7–30 per cent horse serum in PBS and allowed to sediment for 4 hours at 4°. Single fractions (20 ml) were collected and the percentage of lymphoid cells and macrophages was estimated according to morphological criterias. (●) Lymphoid cells; (△) macrophages. Fractions 1–8 were pooled. This cell pool contained 98·5 per cent macrophages and 1·5 per cent lymphoid cells.

survived a consecutive incubation period of 24 hours as functionally active cells is suggested by the results described in Table 2; no loss of cytotoxic activity was observed. The data given in Table 3 show that upon incubation with supernatants derived from cultures containing H-2^k CTL, H-2^k macrophages acquired the capacity to lyse allogeneic target cells. Similar results were obtained with supernatants derived from cultures containing specifically sensitized T cells incubated together with either medium alone or with unrelated target cells (Table 3). However, no 'active' supernatant was produced provided the cells containing CTL were treated with AKR anti-θ serum plus complement prior to the 24-hour incubation period. Since T cells from normal H-2^k splenic lymphocytes failed to produce 'active' supernatant (Table 3), we concluded that production of 'active' super-

TABLE 1
SPECIFIC CYTOTOXICITY OF *in vitro* SENSITIZED CTL

	Percentage lysis of target cells	
CTL	P815 (H-2^d)	EL4 (H-2^b)
C57Bl/6 anti-H-2^d	75·1 ± 5·0	0·5 ± 0·3
DBA/2 anti-H-2^b	5·0 ± 1·5	83·6 ± 2·1

5 x 10^6 Spleen cells were cultured together with 0·5 x 10^6 mitomycin C-treated stimulator spleen cells. After 5 days of culture, 5 x 10^5 viable cells were tested for cytotoxicity (3 hours) against 5 x 10^4 ^{51}Cr-labelled target cells. Spontaneous release of target cells used was less than 12 per cent.

TABLE 2

PERCENTAGE LYSIS OF H-2^d AND H-2^b TARGET CELLS AFTER INCUBATION OF CTL WITH TARGET CELLS

CTL	Percentage lysis of target cells	
	P815 (H-2^d)	EL4 (H-2^b)
C57Bl/6 anti-H-2^d	75·1±5·0	1·2±0·4
C57Bl/6 anti-H-2^d after incubation with P815 cells (24 hours)	98·5±1·5	2·0±0·8
C57Bl/6 anti-H-2^d after incubation with EL4 cells (24 hours)	91·0±3·2	4·1±1·0
C57Bl/6 anti-H-2^d after incubation without cells (24 hours)	83·5±1·8	1·5±0·5

C57Bl/6 mouse-derived spleen cells were sensitized against H-2^d alloantigens as described in the legend to Table 1. 20×10^6 viable cells were incubated for 24 hours with different target cells at a ratio of 100:1. After this incubation period, viability was determined by Eosin dye exclusion, and cytolytic activity of the remaining viable cells was assayed at a ratio of effector cells to target cells of 20:1. Background lysis of target cells was: P815 = 7·5 per cent; EL4 = 9 per cent.

TABLE 3

INDUCTION OF MACROPHAGE-MEDIATED CYTOTOXICITY WITH SUPERNATANTS FROM SPECIFICALLY ACTIVATED CTL

CBA mouse-derived macrophages incubated with supernatants from 24-hour cultures containing:	Percentage lysis of target cells	
	P815 (H-2^d)	EL4 (H-2^b)
CBA anti-H-2^b CTL+EL4	69·5±0·5	63·8±5·2
CBA anti-H-2^b CTL+P815	67·0±7·7	48·7±4·2
CBA lymphocytes, the CTL of which were killed by treatment with AKR anti-θ serum plus complement*	0·7±4·2	4·0±2·1
CBA anti-H-2^d CTL+P815	65·7±7·9	46·7±5·7
CBA anti-H-2^d CTL+EL4	65·0±1·0	59·0±9·3
CBA anti-H-2^d CTL+medium	60·8±6·7	62·5±4·5
Normal CBA spleen cells+P815	0	0·5±1·0
Normal CBA spleen cells	0	1·0±1·0
Medium	0	0

Supernatants derived from cultures containing *in vitro* sensitized CTL incubated either alone or together with allogeneic target cells were filtered through 0·22 μm Millipore filters and diluted 1:4 with culture medium. Two millilitres of diluted supernatant were added to normal macrophage monolayers. After 24 hours the culture medium was removed and ^{51}Cr-labelled target cells were added at a ratio of twenty macrophages to one target cell. After a further incubation of 18 hours, the percentage lysis of the target cells was determined. Background lysis: P815 = 27±2·0 per cent; EL4 = 32·5±3·5 per cent. No lysis was observed above background when active supernatants were incubated with target cells in the absence of macrophages.

* The cells from cultures containing CBA anti-H-2^d CTL were first treated with AKR anti-θ serum (Wagner, 1973) plus complement. The remaining viable cells were used for the production of supernatant.

natant was dependent on the presence of CTL. The results given in Fig. 2 show that under the conditions used, *in vitro* 'activated' macrophages are capable of lysing about 60 per cent of the target cells within 18–24 hours. Therefore, in all further experiments an incubation period of 18 hours was selected for the cytotoxicity assay. It was noted that the cytotoxicity mediated by 'activated' macrophages appeared not to be antigen-specific. These results

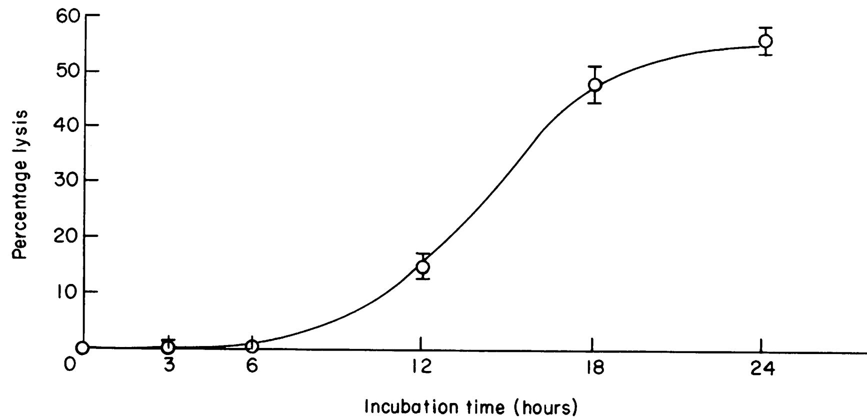

FIG. 2. Kinetics of macrophage-mediated cytotoxicity. CBA mouse-derived macrophage monolayer cultures were incubated (for 24 hours) with 'active' supernatant. Thereafter macrophage-mediated cytotoxicity against P815 target cells (percentage lysis ± s.d.) was recorded over time. Background lysis of P815 target cells (24 hours) was 30 ± 2 per cent.

were obtained both with 'activated' macrophages derived from induced PC as well as from non-induced PC. For example, irrespective of whether the supernatants used for 'activating' syngeneic macrophages were derived from anti-H-2^b or anti-H-2^d sensitized CTL, both H-2^d and H-2^b target cells were lysed (Table 3). Moreover, macrophages were 'activated' equally well by supernatants derived from allogeneic CTL (Table 4). Finally we observed that 'activated' DBA/2 macrophages were also capable of lysing effectively syngeneic P815 target cells (Table 4).

TABLE 4

ACTIVATION OF MACROPHAGES BY SUPERNATANTS FROM SYNGENEIC OR ALLOGENEIC CTL

Macrophages	Supernatants from:	Dilution of 'active supernatant'	Percentage lysis of P815 target cells
C57Bl/6	C57Bl/6 anti-H-2^d CTL	1:4	66·2 ± 3·3
	DBA/2 anti-H-2^b CTL	1:4	76·3 ± 5·3
	CBA anti-H-2^d CTL	1:4	47·3 ± 3·0
		1:40	2·0 ± 2·8
	CBA anti-H-2^b CTL	1:4	48·0 ± 11·5
		1:40	3·5 ± 0·7
DBA/2	DBA/2 anti-H-2^b CTL	1:4	66·0 ± 2·5

For production of 'active supernatant', cultures containing CTL were incubated (24 hours) together with target cells carrying the same alloantigens against which the CTL have been immunized. Macrophages were activated as described in the legend to Table 3. Macrophage monolayers were incubated for 18 hours with P815 target cells at a ratio of 10:1. Background lysis of P815 target cells was 29 ± 3 per cent.

ACTIVATION OF MACROPHAGES BY PRODUCTS OF UNSPECIFICALLY SENSITIZED T CELLS

Since 'active' supernatants produced by specifically sensitized CTL-induced non-specific macrophage-mediated cytotoxicity, it was of interest to investigate whether super-

natants derived from mitogen-stimulated T cells also are capable of rendering macrophages cytotoxic. The data depicted in Table 5 clearly demonstrate that 'active' supernatants are obtained both from Con A- and PHA-induced T-cell blasts. In contrast, supernatants obtained from LPS-stimulated spleen cells (B cell blasts), fibroblasts or tumour cells failed to render macrophages cytotoxic.

TABLE 5

ACTIVATION OF C57Bl/6 MOUSE-DERIVED MACROPHAGES BY FACTOR(S) RELEASED FROM MITOGEN-STIMULATED T CELLS

Supernatants from:		Percentage lysis of target cells	
Cells	Mice	P815 (H-2^d)	EL4 (H-2^b)
Con A blast cells	C57Bl/6	52·3±4·1	54·5±2·0
	CBA/J	64·1±5·2	60·7±5·6
	BALB/c	30·3±2·0	17·8±2·4
PHA blast cells	DBA/2	40·9±9·8	n.d.
LPS blast cells	C57Bl/6	0	0
	CBA/J	2·1±1·5	n.d.
	BALB/c	−1	n.d.
	DBA/2	0·5±2·0	n.d.
Fibroblasts	C57Bl/6	1·5±0·9	n.d.
P815		0	n.d.
EL4		0	n.d.
Medium+Con A (2·5 μg/ml)		−2	1·5±1·0

n.d. = Not determined.

Mouse-derived splenic lymphocytes were stimulated by either PHA, Con A or LPS (3-day cultures). The stimulated cells were harvested, washed twice and recultured for 24 hours. Supernatants from PHA, Con A or LPS-stimulated lymphocytes (24-hour period) were used to 'activate' macrophage cultures. Supernatants of embryonal fibroblasts, suspension culture of P815 and EL4 were also tested. The experimental protocol used was identical to that given in the legend to Table 3. Background lysis of the ^{51}Cr-labelled target cells (18 hours) was less than 33 per cent.

TABLE 6

KINETICS OF MACROPHAGE ACTIVATION

Incubation time (hours)	Percentage lysis of P815 target cells
20	77·2±3·4
10	16·4±2·7
5	3·0±1·5
2·5	0·5±1·0
1	2·0±1·0
0·5	2·0±1·5
0	0

CBA mouse-derived macrophage monolayer cultures were incubated with 'active' supernatant for the time intervals given. At the time point zero all monolayer cultures were washed and ^{51}Cr-labelled target cells (ratio 10:1) were added. After 18 hours of incubation, the percentage lysis of the target cells was determined. Background lysis of the target cells was 25±3 per cent.

KINETICS OF MACROPHAGE ACTIVATION

Assuming that the 'activation' phase of macrophages represents a mere binding of T-cell

products, one would predict that the 'activation' period takes place within minutes. On the other hand, if the 'activation phase' implies a time-consuming metabolic 'activation' of macrophages, the time period required for effective 'activation' of macrophages would be longer. The results given in Table 6 indicate that an incubation period of less than 20 hours was suboptimal for *in vitro* activation of macrophages. Thus, the results favour the second alternative.

DISCUSSION

There is evidence that macrophages from alloimmune mice and macrophages 'activated' *in vitro* by products of CTL are capable of exerting specific cytotoxic effector functions (Evans and Alexander, 1972b; Evans, Cox and Alexander, 1973; Lohmann-Matthes *et al.*, 1972; Ziegler, Lohmann-Matthes and Fischer, 1975). Since in our hands macrophages obtained from alloimmune mice did not exhibit antigen-specific cytotoxicity (K. Pfizenmaier, manuscript in preparation) we investigated whether 'factors' produced in cultures containing highly active CTL would render normal macrophages specifically cytotoxic. The data presented here support three main conclusions. In the course of *in vitro* cytotoxic mouse allograft responses, adherent cells (macrophages) are capable of acting as cytotoxic effector cells. Secondly, the activation of macrophages can be induced by factor(s) derived from specifically or non-specifically stimulated T cells. Thirdly, the macrophage-mediated cytotoxicity appears to be non-specific, that is, target cells of different H-2 haplotypes are lysed equally well. Thus, on the basis of the specificity of the cytotoxic reaction, the non-specific macrophage-mediated cytotoxicity can be distinguished from the antigen specific cytotoxicity as mediated by CTL.

Because cells resembling macrophages are found in abundant numbers in skin allografts, graft-versus-host reactions and renal allografts (Giroud, Spector and Willoughby, 1970) a critical role of such cells has been postulated in tissue and organ graft rejection. Indeed, both in syngeneic tumour systems and in allograft situations several groups of investigators have provided evidence that macrophages do act as cytotoxic effector cells *in vitro* (Evans and Alexander, 1972a, b; Lohmann-Matthes and Fischer, 1973; Hibbs *et al.*, 1972). Equivocal evidence, however, is available in regard to the specificity of the macrophage 'activation' and to the specificity of the macrophage-mediated cytotoxicity (Granger and Weiser, 1966; Hibbs *et al.*, 1972; Keller, 1973; Den Otter, Evans and Alexander, 1972; Lohmann-Matthes *et al.*, 1972; Lohmann-Matthes and Fischer, 1973; Zbar, Wepsic, Borsos and Rapp, 1970). This may be in part due to the fact that the assay systems used for detecting macrophage cytotoxicity range from long-term target cell 'inhibition' assays (Evans and Alexander, 1972a; Gallily, 1975) to direct cytotoxicity assays (Lohmann-Matthes *et al.*, 1972; Dimitriu *et al.*, 1975).

Using a short-term ^{51}Cr assay (3–6 hours), we have repeatedly failed in the past to detect macrophage-mediated cytotoxicity (Wagner *et al.*, 1973; Röllinghoff *et al.*, 1974). In the present study, we therefore adapted the experimental protocol of Lohmann-Matthes *et al.* (1973) in order to test, whether products obtained from cultures containing specifically sensitized T cells are capable of rendering macrophages specifically cytotoxic. Here, the macrophage cytotoxicity was tested in a long-term (18 hours) ^{51}Cr assay.

The results obtained clearly confirm the original observation of Evans and Alexander (1973) and Lohmann-Matthes *et al.* (1973; Lohmann-Matthes and Fischer, 1973), namely that supernatants derived from cultures containing specifically activated T cells are

capable of 'activating' macrophages to efficient cytotoxic effector cells (Tables 3 and 4). However, the results presented fail to demonstrate antigen-specific macrophage-mediated cytotoxicity. In our experiments (n = 12) there was no exception to this lack of specificity. In addition, the data given here suggest that specifically activated T cells do not require specific restimulation in order to produce 'active' supernatants (Table 3). Finally, mitogen-activated T cells were also found to be capable of producing 'active' supernatant (Table 5). On the basis of these data we conclude, that as a consequence of a specific or non-specific stimulation of T cells, factors are released, which in turn are capable of 'activating' macrophages to non-specific cytotoxic effector cells (Tables 3, 4 and 5).

The term 'macrophage arming factor' (MAF) has been used to indicate that specifically sensitized T cells are capable of 'arming' normal macrophages and rendering them specifically cytotoxic (Evans and Alexander, 1973; Lohmann-Matthes *et al.*, 1973; Lohmann-Matthes and Fischer, 1973; Ziegler *et al.*, 1975). On the basis of this concept it has been postulated that MAF may represent a specific T-cell product (receptors). According to this view the specificity of the macrophage-mediated cytotoxicity is dictated by the T-cell factor (receptor) bound to the macrophage cell surface. The data represented here and by others (Dimitriu *et al.*, 1975; Keller, 1973), however, suggest that factors produced by both specifically and non-specifically activated T cells induce a non-specific macrophage-mediated cytotoxicity. Thus, our data are compatible with the concept that the factors released by activated T cells may represent non-specific lymphokine-like substance(s) capable of 'activating' macrophages non-specifically to non-specific cytotoxic effector cells.

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