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The induction of lymphocytes with the capacity to render macrophages cytotoxic in an allogeneic murine system

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Summary. Sensitized spleen and peripheral lymph node lymphocytes were tested after different types of immunization with allogeneic tumour cells for their capacity to induce macrophage cytotoxicity *in vitro*. The macrophages were rendered cytotoxic either by direct contact with lymphocytes and tumour cells (*activation of macrophages*) or by a factor (macrophage arming factor, MAF), released by the sensitized lymphocytes incubated with tumour cells (*arming of macrophages*). Both types of reactions are T-cell dependent. Macrophage activation is a more sensitive way to detect lymphocytes with the capacity to render macrophages cytotoxic than arming of macrophages. The route of immunization subcutaneously (s.c) or intraperitoneally (i.p.) with allogeneic cells did not influence the induction of lymphocytes with the capacity to render macrophages cytotoxic. However, the tumour cells had to be intact as disrupted cells (suspended in Freund's complete adjuvant, FCA) did not induce macrophages activating lymphocytes. The adjuvant dimethyl dioctadecyl ammonium bromide (DDA) did not increase the lymphocyte response. Intact allogeneic tumour cells were needed *in vitro* when used for secondary antigenic stimulation. This secondary stimulation was independent of antigen presentation by macrophages. This suggests that also *in vivo* the primary response is independent of macrophage antigen presentation.

Delayed-type hypersensitivity and antibody responses against the allogeneic tumour cells were comparable after s.c. and i.p. immunization and after immunization with FCA and DDA.

Abbreviations: CI, cytotoxicity index; DDA, dioctadecyl diammonium bromide; DTH, delayed-type hypersensitivity; FBS, foetal bovine serum; FCA, Freund's complete adjuvant; i.p., intraperitoneal(ly); PBS, phosphate-buffered saline; P(E)C, peritoneal (exudate) cells; PLN, peripheral lymph nodes; s.c., subcutaneous(ly); MAF, macrophage arming factor.

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INTRODUCTION

In studies concerning lymphocyte-induced macrophage cytotoxicity in allogeneic murine models different types of immunization have been used, such as subcutaneous (Fidler, Darnell & Budmen, 1976) and intraperitoneal sensitization (Evans & Alexander, 1972a, 1972b; Lohmann-Matthes, Ziegler & Fischer, 1973; Pels & Den Otter, 1974) *in vivo* and different types of *in vitro* stimulation of the lymphocytes, such as triggering by the specific antigen (Lohmann-Matthes *et al.*, 1973; Pels & Den Otter, 1974) or aspecific (mitogen) stimulation (Fidler *et al.*, 1976; Piessens, Churchill & Sharma, 1981). No detailed information is available however which type of immunization and triggering favours the development

and detection of lymphocytes able to render macrophages cytotoxic *in vitro*. These lymphocytes were described to be T lymphocytes (Evans & Alexander, 1972a, 1972b) and therefore immunizations which induce good delayed-type hypersensitivity (DTH) reactions seem to be favourable. Sensitizations favouring DTH reactions are subcutaneous immunizations and the use of adjuvants (Crowle, 1975). Smith & Miller (1979a, 1979b) have determined the optimal conditions for DTH reactions in allogeneic sensitization models. It was shown that secondary reactions to alloantigens depend on the type of primary sensitization. Secondary reactions of T cells, sensitized to allogeneic cells, is independent of macrophages when triggered by the same allogeneic cells. T cells sensitized to solubilized alloantigens, however, need to be triggered by syngeneic macrophages fed with the alloantigen (Smith & Miller, 1979b; Mottram & Miller, 1980).

In this study the capacity of lymphocytes to induce macrophage cytotoxicity is tested after different types of immunization with alloantigens. The induction of these lymphocytes is shown to be dependent of immunization with intact cells and is independent of antigen presentation by macrophages.

MATERIALS AND METHODS

Animals and tumour

Inbred C57BL/10 mice (6–10 weeks, female, Bomholtgård, Denmark), and inbred CBA and inbred DBA/2 mice (6–10 weeks, female and male, TNO, Zeist Holland) were used.

The DBA/2 lymphoma SL2 grew as ascitic tumour when transplanted intraperitoneally (i.p.). The tumour was maintained by weekly i.p. passage.

Immunization

Four immunization procedures were tested (C57BL mice were injected): (i) intraperitoneally (i.p.) with 10^7 SL2 cells in 1 ml of phosphate-buffered saline (PBS; i.p. immunization); (ii) subcutaneously (s.c.) at two sites in the chest with 10^7 SL2 cells in 0·1 ml of PBS (s.c. immunization); (iii) s.c. at two sites on the chest with 0·1 ml of a mixture of 0·05 ml 2×10^7 SL2 cells and 0·05 ml Freunds complete adjuvant (FCA; FCA immunization); (iv) sc. at two sites in the chest with 0·1 ml of PBS containing 10^7 SL2 cells and 100 μg dioctadecyl diammonium bromide (DDA; DDA immunization).

Histology

Nodules, which developed at the immunization site on the chest were removed and fixed in 4% formaldehyde and embedded in paraffin. Tissue sections were stained with haematoxylin/eosin. Peritoneal exudates were studied as described previously (Pels & Den Otter, 1979).

Cell cultures

Foetal bovine serum (FBS) and Fischer's medium were obtained from Flow Laboratories. Growth medium was Fischer's medium supplemented with 10% FBS.

Tumour cell cultures. The SL2 tumour cells were harvested 10 days after i.p. transplantation, washed in Fischer's medium and suspended at a concentration of $1{\cdot}5–2 \times 10^5$ cells/ml in growth medium.

Lymphocyte cultures. The lymph nodes were squeezed through a metal sieve in medium. The cell suspension was centrifuged and the cell pellet was resuspended. After removal of the dead cells and cell debris by glass wool filtration the lymphocytes were resuspended in growth medium (2×10^6 cells/ml). The spleens were perfused with 10 ml of medium to remove the lymphocytes. The cells were spun down and suspended in growth medium (2×10^6 lymphocytes/ml).

Macrophage cultures. Peritoneal cells (PC) were collected from the unstimulated peritoneal cavity of normal C57BL mice. PC containing 7×10^5 macrophages were seeded into culture dishes (Costar, Ø 16·0 mm.). The macrophages were allowed to adhere at 37° in a humidified atmosphere containing 5% CO_2 in air. After 1 hr medium was removed and fresh Fischer's medium was added to the culture. The cultures were incubated overnight and before use washed with jets of medium from a Pasteur pipette to remove non-adhering cells. The adhering cells formed a confluent monolayer. At least 97% of the cells were characterized as macrophages as described previously (Pels & Den Otter, 1979).

Production of macrophage arming factor

Lymphocytes (2×10^6 lymphocytes/ml) were mixed with SL2 tumour cells (2×10^5 cells/ml) in a ratio 2:1 (v/v) and incubated at 37°. After 24 hr the supernatant was tested for macrophage arming activity (MAF).

Arming of macrophages
Macrophage monolayers were incubated with 0·6 ml of supernatant for 4 hr (arming). After incubation the armed macrophages were washed with medium and challenged with 0·6 ml of a suspension of 10^5 SL2 tumour cells/ml.

Activation of macrophages
Lymphocytes (2×10^6 cells/ml) were mixed with tumour cells (2×10^5 cells/ml) in a ratio of 2:1 (v/v). This cell mixture (0·9 ml) was added to the macrophage monolayer for 24 hr (activation). After incubation the lymphocytes and tumour cells were washed from the monolayer and challenged with 0·6 ml of a suspension of 10^5 SL2 tumour cells/ml.

Cytotoxicity
Cytotoxicity was assessed after 24 hr comparing the growth of tumour cells in the test system with the growth of tumour cells in the control. In control experiments the sensitized lymphocytes were replaced by lymphocytes from normal mice.

Before counting, the cultures were incubated with 10 μl indian ink (1/10 diluted) for half an hour. The macrophages phagocytose indian ink, the tumour cells do not. The tumour cells were counted in a haemocytometer by phase contrast microscopy. Only viable tumour cells were counted as judged by trypan blue exclusion. Cytotoxicity was expressed as: $CI = (1 - T/N) \times 100$. Where CI is the cytotoxicity index, N is the number of tumour cells in controls, and T is the number in the test system.

Treatment of lymphocytes with anti-T-cell serum or anti-Thy-1·2 serum
One volume of lymphocyte suspension was incubated with one volume of rabbit anti-mouse thymocyte serum (1/250, Nutacon), or AKR anti-Lyt-1·2 serum (1/2·5, Bionetics, batch number CC047) for 30 min at 4°. After incubation one volume 1/10 diluted guinea-pig complement (RIV-Bilthoven, Holland) was added, and the suspension was incubated at 37° for 30 min. After treatment the cells were washed and resuspended in growth medium.

Elimination of T cells in the monolayers
After activation monolayers were incubated with 1 ml of rabbit anti-mouse T-cell serum (Bionetics), 1/500 diluted, at 4° for 30 min. One millilitre of guinea-pig complement (1/10 diluted) was added and the monolayers were incubated at 37° for 30 min. After treatment the monolayers were washed.

Antibody titration
At several days after immunization mice were bled from the orbital venous plexus. Sera were decomplemented by incubation at 56° for 30 min and tested. Serial dilutions of the sera were made in microtitre plates. Fifty microlitres of serum dilution were incubated with 0·05 ml SL2 suspension (3×10^6 cells/ml) and 0·05 ml 1/10 guinea-pig complement. After 1 hr of incubation at 37° the SL2 cells were counted in a haemocytometer. The titre was considered as the highest serum dilution which caused fifty percent lysis of the SL2 cells.

In some experiments serum was added to normal macrophage monolayers for 1 hr at 4°. After washing the monolayers were challenged and the cytotoxicity was determined.

Foodpad swell test
The increase in foodpad thickness in C57BL mice (foodpad swell test) was measured with a foodpad meter as described by Kerchhaert, Van Der Berg & Willers (1974) at different days after immunization. The eliciting dose was given by an injection into the left foodpad of 4×10^6 SL2 cells suspended in 0·05 ml PBS. The right foodpad was only injected with 0·05 ml PBS. The results were expressed as the mean of the foodpad thickness of five mice after 24 hr. The mean $\pm$ SD of foodpad swelling elicited with 4×10^6 SL2 cells in normal animals was $0{\cdot}08 \pm 0{\cdot}12$ mm. A foodpad swelling of $>0{\cdot}32$ mm was considered significant ($P < 0{\cdot}025$).

RESULTS

Activating and arming capacities of sensitized lymphocytes

Lymphocytes (spleen or peripheral lymph nodes; PLN) of C57BL mice were collected at different days after immunization with: (i) 10^7 allogeneic SL2 cells s.c. (s.c. immunization); (ii) 10^7 SL2 cells mixed with 100 μg DDA s.c. (DDA immunization); (iii) 10^7 SL2 cells mixed with FCA (FCA immunization); (iv) 10^7 SL2 cells i.p. (i.p. immunization). The lymphocytes were tested for their capacity to activate macrophages by direct contact with the macrophages and tumour cells and for their capacity to produce macrophage arming factor (MAF) after a secondary stimulation with SL2 cells *in vitro* (Fig. 1).

After s.c. and DDA immunization lymphocytes with activation capacity were present in the draining PLN from 4 to 15 days and in the spleen from 4 to 20

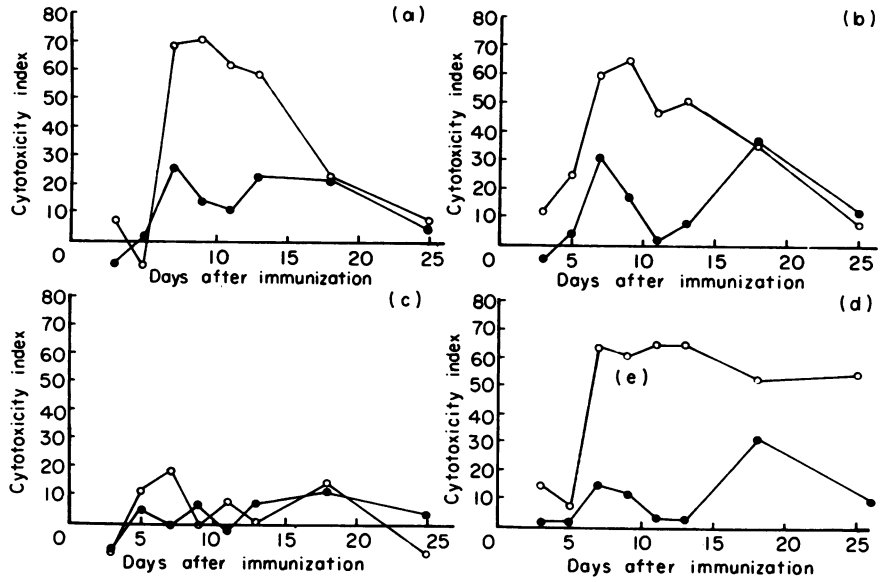

Figure 1. Kinetics of MAF production and activation capacity of spleen lymphocytes after immunization with allogeneic tumour cells. Mean of a representative of five experiments in triplicate. (O——O) Cytotoxicity index (CI) of C57BL macrophages incubated with immune spleen lymphocytes and SL2 cells for 24 hr (activated macrophages); (●——●) CI of C57BL macrophages incubated (4 hr) with the supernatant of immune spleen lymphocytes, cultured with SL2 cells for 24 hr (armed macrophages). Lymphocytes collected at different days after (a) s.c. immunization, (b) DDA immunization, (c) FCA immunization, (d) i.p. immunization. The CI was assessed against SL2 cells 24 hr after challenge. The SD of the mean values was 15%.

days. MAF-producing lymphocytes were also present from 4 to 15 days after immunization in the draining PLN. The MAF production by spleen lymphocytes showed a biphasic pattern as activity was found 5–10 days and 15–20 days after immunization. After i.p. immunization the MAF producing and activating activity of the spleen lymphocytes showed basically the same kinetics as after s.c. immunization. The only difference was that the activating capacity of the lymphocytes was preserved some days longer.

Table 1. Effect of T-cell elimination on the MAF producing and activating capacities of immune PLN lymphocytes

Treatment of the lymphocytes*	Cytotoxicity index†	
	MAF armed macrophages	Activated macrophages
No treatment	38±3	59±6
Rabbit anti-T-cell serum and complement	−5±8	10±8
AKR anti-Thy-1·2 serum and complement	−7±10	10±9

Mean±SEM of three experiments in triplicate.

* Lymphocytes obtained 7 days after s.c. immunization were tested for their MAF producing or activating capacity with or without previous treatment with anti-T-cell serum (1/250) or anti-Thy-1·2 (1/2·5) serum and complement (1/10).

† Measured versus SL2 cells 24 hr after challenge.

No activation of MAF production could be shown after FCA immunization. The cytotoxicity of MAF-armed macrophages was always less than the cytotoxicity of activated macrophages. Activation of the macrophages or production of arming factors could not be induced with sensitized lymphocytes in the absence of SL2 cells.

As shown in Table 1, treatment of sensitized lymphocytes with anti-Thy-1·2 serum, or anti-T-cell serum and complement resulted in abrogation of both the activating and MAF producing capacity of the lymphocytes.

Suppressive effect of lymphocytes after FCA immunization

As indicated after FCA-immunization, lymphocytes could not activate macrophages. To test whether the macrophage activating lymphocytes were inhibited by suppressor cells after FCA immunization, lymphocytes from mice after s.c. immunization were mixed with lymphocytes from mice after FCA immunization. As shown in Table 2, lymphocytes obtained after FCA immunization could not inhibit the activation of macrophages by lymphocytes obtained after s.c. immunization.

Table 2. The influence of spleen and PLN lymphocytes after FCA immunization on the activating capacity of immune lymphocytes after s.c. immunization

Composition* of 'activating' lymphocytes			CI macrophages¶ after 'activation' with lymphocytes from	
FCA†	SC‡	Normal§	Spleen	PLN
—	—	$1·2 \times 10^6$	0	0
—	—	$0·6 \times 10^6$	−4±2	−5±9
—	$1·2 \times 10^6$	—	55±6	56±4
—	$0·6 \times 10^6$	—	51±3	59±1
—	$0·6 \times 10^6$	$0·6 \times 10^6$	47±5	54±3
$0·6 \times 10^6$	$0·6 \times 10^6$	—	41±12	53±6
$0·6 \times 10^6$	—	$0·6 \times 10^6$	8±5	6±3
$0·6 \times 10^6$	—	—	10±7	−3±9
$1·2 \times 10^6$	—	—	4±3	9±6

Mean ± SEM of three experiments in triplicate.
* Number of C57BL lymphocytes mixed with 6×10^4 SL2 cells in 0·9 ml growth medium.
† Lymphocytes obtained 7 days after FCA immunization.
‡ Lymphocytes obtained 7 days after SC-immunization.
§ Lymphocytes obtained from normal C57BL mice.
¶ 'Activated' C57BL macrophages CI versus SL2 cells, assessed 24 hr after challenge.

Table 3. Genetic restriction of macrophage arming and activation

Type of macrophage	Presence of SL2*	Cytotoxicity index	
		Arming	Activation
C57BL	—	0±3	−3±4
C57BL	+	42±6	62±5
CBA	—	1±2	8±4
CBA	+	33±5	48±3

Production of MAF and activation by C57BL immune PLN lymphocytes 8 days after s.c. immunization.
* Factor production or activation of macrophages by immune C57BL lymphocytes in the presence (+) or absence (−) of SL2 cells.
† Mean ± SEM of three experiments in triplicate.

Treatment of activated macrophage monolayers with anti-T-cell serum

Treatment of activated macrophage monolayers with anti-T-cell serum and complement did not reduce the cytotoxicity. This indicated that adhering T cells were not responsible for the cytotoxicity.

Genetic restriction of macrophage activation and arming

The arming of macrophages was not genetically restricted as both C57BL and CBA macrophages were armed by the factor produced by C57BL sensitized lymphocytes (Table 3). To test whether antigen presentation by macrophages was necessary, lymphocyte populations were depleted of macrophages by nylon wool adherence or glass adherence (2 hr, 37°). MAF production was not reduced after these treatments. Activation was not genetically restricted as (macrophage depleted) C57BL sensitized lymphocyte populations (obtained after s.c. immunization) were able to activate both C57BL and CBA monolayers (Table 3).

Humoral response after immunization with allogeneic tumour cells

Serum collected at different days after immunization

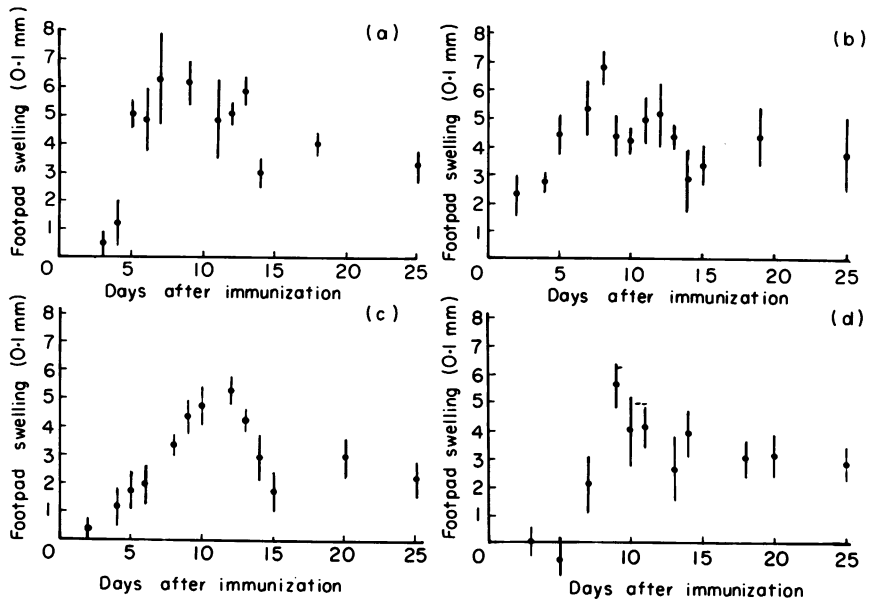

Figure 2. Kinetics of DTH reaction after immunization with allogeneic tumour cells measured by the footpad swell test. Mean ± SEM of three to individual experiments done in five-fold C57BL mice were stimulated in the left footpad with 4×10^6 SL2 cells after (a) s.c. immunization, (b) DDA immunization, (c) FCA immunization, (d) i.p. immunization. The increase in footpad thickness was measured after 24 hr. A footpad swelling >0·32 mm was considered significant ($P < 0.05$).

were tested for their lytic activity in the presence of complement for SL2 cells. Only low titres were found after all four types of immunization. The highest titre (1/128) was found after s.c. immunization between 10 and 14 days. When the sera were added (diluted 1/10, 1/20, 1/40) to normal macrophage monolayers, no significant macrophage cytotoxicity was induced (CI < 10).

Foodpad swelling test

To study the kinetic pattern of the delayed-type hypersensitivity reactions *in vivo*, the increase in footpad thickness after stimulation with SL2 cells was measured at different days after immunization. After s.c. immunization (Fig. 2a) and DDA immunization (Fig. 2b) the reactivity started at 5 days, increased quickly with maximal activity at days 7–9, and declined slowly thereafter. The results with FCA immunization showed a comparable pattern (Fig. 2c), but maximal activity was reached after 10–12 days after immunization. Also the increase in foodpad swelling was smaller compared with the swelling after s.c. and DDA immunizations. The foodpad swelling test after i.p. immunization showed a rapid increase of DTH reactivity after 7–8 days, with a maximum reactivity at day 9 (Fig. 2d).

The yield of lymphocytes from the draining PLN and spleen was not different between s.c., i.p., DDA and FCA immunization. A maximum yield, up to four–six times the yield of normal PLN and two–four times the yield of normal spleen, was found 8 days after immunization.

Histological examination of the site of immunization

SL2 cells injected s.c. (s.c. and DDA immunization) showed an initial growth. In C57BL mice nodules developed showing histologically intact tumour cells surrounded by large numbers of mononuclear cells 3–10 days after tumour injection. Necrosis was found centrally (Fig. 3). After 10 days the nodules regressed rapidly and after 15–20 days nodules were no longer palpable. Also after i.p. immunization the tumour cells showed an initial growth, followed by a gradual increase in the number of mononuclear cells. After 8–10 days the number of tumour cells decreased rapidly, while the number of exudate cells decreased

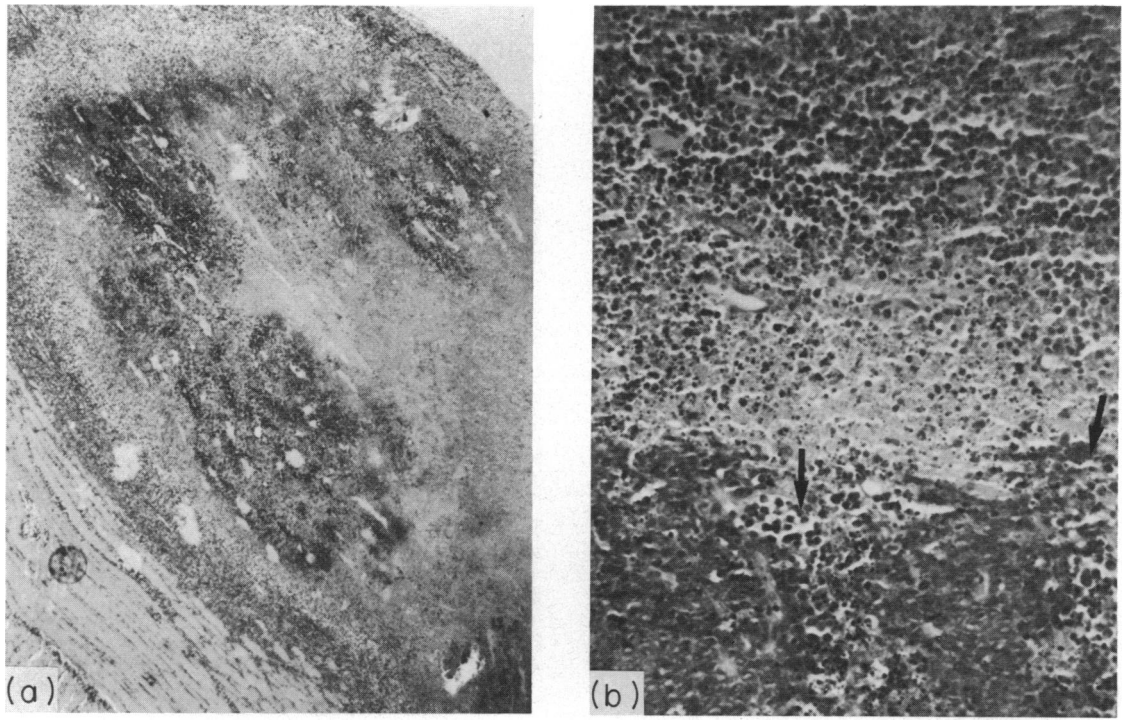

Figure 3. Nodule which developed 10 days after s.c. immunization with 10^7 SL2 tumour cells. (a) Areas of tumour cells (dark) surrounded by necrosis and mononuclear cells (magnification ×40). (b) Mononuclear cells (top) and necrosis (light area) surrounding the tumour cells (below). Some mononuclear cells are penetrating the tumour cell area (arrows; magnification ×200).

slowly after 10–12 days. In the nodules which developed after FCA immunization no intact tumour cells were detected. Small granulomata composed of mononuclear cells, and oil droplets surrounded by mononuclear cells were found (Fig. 4). These nodules regressed after about 15 days.

DISCUSSION

The capacity of *in vivo* allosensitized lymphocytes to render macrophages cytotoxic by direct incubation with the macrophages (activation) or indirectly by the production of a factor (arming) were comparable. This is in agreement with the suggestion of Evans *et al.* (1972b) that both forms of lymphocyte-induced macrophage cytotoxicity are related.

The induction of both armed and activated macrophages was T-cell dependent as T-cell elimination by antisera abrogated the response, whereas nylon wool filtration (B-cell depletion) did not. Furthermore, antibodies were detected in the sera after the different types of immunization, but these sera were not able to induce macrophage cytotoxicity which makes the involvement of antibodies unlikely. The induced cytotoxicity was a true macrophage cytotoxicity and not due to adherent T cells as treatment of the activated monolayers with anti-T-cell serum and complement did not reduce the cytotoxicity. After s.c., i.p. and DDA immunization the presence of lymphocytes able to render macrophages cytotoxic followed a similar pattern. The only difference between activation and arming was that spleen lymphocytes had a biphasic pattern of factor production, which is not found by activation. This biphasic pattern may be compared with the biphasic pattern of MIF production in a regressor tumour system as described by Landolfo, Herberman & Holden (1977). This decrease in MIF production was caused by suppressor macrophages present in the immune spleen-cell population.

After s.c., i.p. and DDA immunization the development of DTH reactions was the same. DDA was

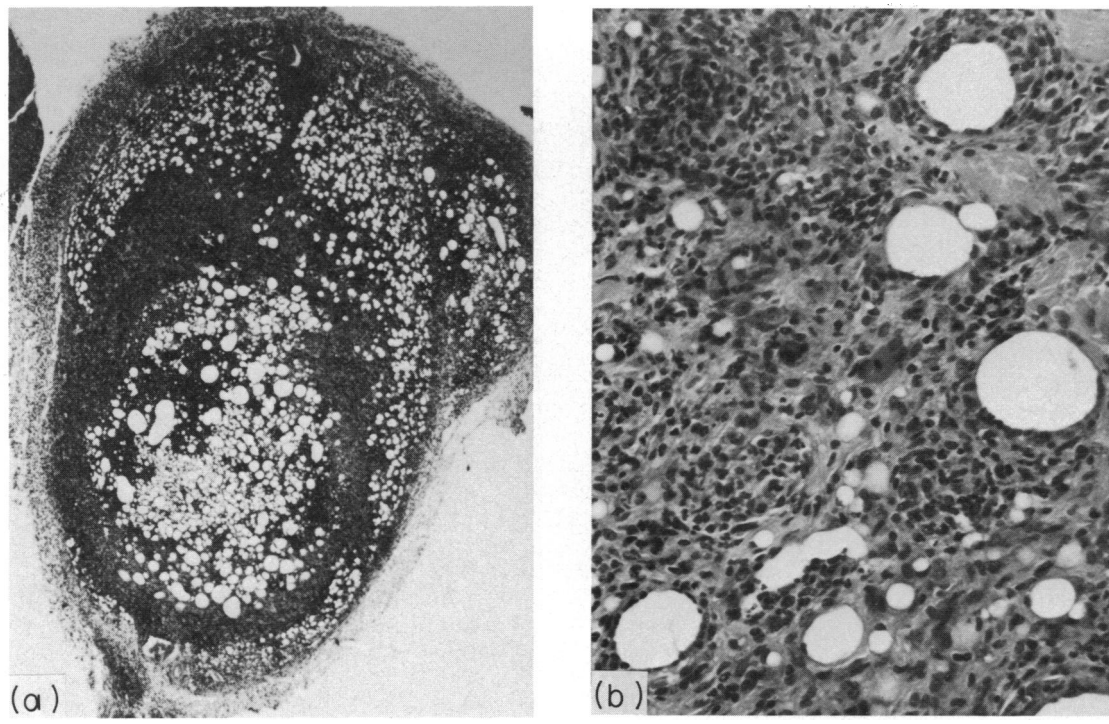

Figure 4. Nodule which developed 10 days after immunization of SL2 cells suspended in FCA. (a) Mononuclear cells surrounding small oildroplets (magnification ×40). (b) Oildroplets surrounded by mononuclear cells and granulomata composed of mononuclear cells (magnification ×400).

described to enhance the T-cell-mediated reactions (Snippe, Belder & Willers, 1977). It was suggested that the effect of DDA was mediated by macrophages, as DDA inhibited the phagolysosome formation and in this way inhibited the digestion of the antigen and favoured its presentation (Hofhuis, Van der Meer, Kersten, Rutten & Willers, 1981). In this study neither the capacity of the lymphocytes to render macrophages cytotoxic, nor the DTH to the alloantigens was enhanced by DDA. This indicated that macrophages are not involved in the lymphocyte sensitization against allogeneic tumour cells.

After FCA immunization neither MAF producing nor macrophage activating lymphocytes could be found. On the other hand DTH, increase in lymphocyte numbers in spleen and lymph node as well as the antibody responses developed normally, although the optimal DTH reaction was present some days later than after s.c., i.p. and DDA immunization and the maximal footpad swelling was relatively small. The absence of lymphocytes able to render macrophages cytotoxic after FCA immunization was not due to suppressor cells. So, after FCA immunization no macrophage activating lymphocytes are present. The changes in numbers of lymph node and spleen lymphocytes did not differ between s.c., i.p., DDA and FCA immunization, indicating that after all types of immunization a good lymphocyte response developed. After DDA and s.c. immunization the mice developed large nodules at the site of immunization composed of tumour cells surrounded by a mononuclear infiltrate and after i.p. immunization an initial growth of the tumour cells was observed, followed by an influx of mononuclear cells. The initial growth of the tumour cells supplied a large amount of cells to be rejected which seems to favour induction of the arming and activating capacity of lymphocytes. In contrast, after FCA immunization the mice developed nodules which did not contain tumour cells, as the cells are disrupted during mixing with the FCA and the alloantigens have to be presented by the macrophages to induce a lymphocyte response. Antibody and (suboptimal) DTH response will develop after this form of sensitization but macrophage activating lymphocytes are not induced.

Macrophage activating and arming were not genetically restricted as both C57BL and CBA macrophages could be activated and armed by the same lymphocytes to the same level. Secondary stimulation by antigen presenting macrophages does only occur when antigen is presented by macrophages bearing the same I-region coded antigens as the macrophages sensitizing the lymphocytes *in vivo* (Smith & Miller, 1976b). As activation of macrophages is not genetically restricted, antigen presentation is not required for secondary stimulation of the macrophage activating lymphocytes. Likewise, the production of MAF by macrophage depleted lymphocyte populations was not reduced which makes antigen presentation by macrophages also unlikely. Perhaps also the sensitization *in vivo* is not dependent on macrophage antigen presentation, but the lymphocytes which render macrophages cytotoxic react directly to the intact tumour cells. This is in agreement with the presence at the site of immunization of intact tumour cells after s.c., i.p. and DDA immunization and the absence of intact tumour cells after FCA immunization.

In conclusion: DTH reactions and antibody responses will develop after immunization with intact and disrupted cells but intact cells induce a better response and the H-2 antigen sensitization is strong enough to obviate the need of adjuvant (Smith & Miller, 1979a, 1979b). Disrupted cells are however, unable to induce macrophage activating lymphocytes. This suggests that only when lymphocytes are sensitized by intact allogeneic cells they are able to render macrophages cytotoxic but not when sensitized by alloantigens presented by macrophages. This suggestion is supported by the observations that living and multipying cells/organisms are necessary for a good immunity to tumour cells (Prager & Baechtel, 1973) and parasites (Patterson & Youmans, 1970), and for the induction of cytolytic peritoneal macrophages (Den Otter, Evans & Alexander, 1974).

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