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Mechanisms of Target Cell Destruction by Alloimmune Peritoneal Macrophages

I. THE EFFECT OF TREATMENT WITH SODIUM FLUORIDE

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Summary. Sodium fluoride (NaF), an inhibitor of membrane activity, was used to investigate the mechanisms by which alloimmune macrophages kill target cells and produce plaques in monolayers of specific target cells. When target cells were treated with 0·02 M or 0·002 M NaF and washed before adding alloimmune macrophages to establish loci of cell interaction, plaque formation was not affected. However, when interacting alloimmune macrophages and target cells were initially exposed for 30 minutes or 1 hour to 0·02 M or 0·002 M NaF plaque formation was markedly suppressed. Comparative studies with NaF and the inhibitor of protein synthesis, Puromycin, showed that protein synthesis by macrophages was not affected by treatment with 0·002 M NaF. Results obtained by subjecting interacting cells to 1 hour exposure to NaF at various intervals indicated that the destruction of target cells by immune macrophages was irreversibly initiated in large measure during the first hour of cell interaction. It is postulated that the membrane activity of immune macrophages responsible for target cell killing involves the release or secretion of a cytoxin.

INTRODUCTION

Gorer (1956) was the first to suggest that the macrophage is important in allograft rejection. He observed that macrophages accumulated in response to allografts of certain tumours and appeared to destroy them. The findings of Old, Boyse, Bennett and Lilly (1963) and Weiser and his colleagues (Granger and Weiser, 1964, 1966; Tsoi and Weiser, 1968; Pearsall and Weiser, 1968a, b) have established that immune macrophages function as effector cells in allograft systems of mice, both *in vitro* and *in vivo*.

Granger and Weiser (1964, 1966) showed that alloimmune peritoneal macrophages of the mouse can cause specific destruction of target cells *in vitro* by a non-phagocytic contact mechanism independent of complement. The initial step in the reaction, the specific adherence of immune cells to target cells, was found to depend on cytophilic antibody. Presumably adherence itself does not cause injury, but instead provides the opportunity for some additional event which results in target cell death. The results of Weiser, Heise, McIvor, Granger and Han (1969) indicate that the event subsequent to adherence is specific. Moreover, they showed that the supernatant from mixed cultures of immune macrophages and target cells collected after 2 hours of incubation contains a growth inhibitory factor (GIF) for target cells.

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The present report concerns initial investigations designed to identify the specific event, occurring subsequent to cell adherence, that leads to target cell killing.

MATERIALS AND METHODS

Unless specified otherwise, the following materials and methods were used.

Animals. Our stock colonies of inbred A/Jax and C57BL/Ks mice are periodically renewed from offspring of constant brother–sister matings. They retain syngrafts of skin permanently. The animals used were from 2 to 6 months old and were maintained on commercial mouse breeder pellets and water.

Tissue culture media. A supplemented Eagle's minimal essential medium (SMEM) (Grand Island Biological Supply, Oakland, California) was commonly used for cell collection and culture. Supplementation was as follows: 200 mg glutamine/litre; 2240 mg sodium bicarbonate/litre; 100 units penicillin/ml and 100 μg streptomycin/ml; and 10 per cent new-born calf serum (Hyland Laboratories, Los Angeles, California.) The Hanks's balanced salt solution used was supplemented with 0·1 per cent yeast extract (Difco Laboratories, Detroit, Michigan); 0·01 per cent proteose peptone No. 3 (Difco Laboratories); 100 units penicillin/ml; 100 μg streptomycin/ml; and 10 per cent new-born calf serum (SHBSS). Phenol red was added as an indicator at a final concentration of 0·02 per cent.

Cell counts. Total cell counts and viable cell counts using 0·02 per cent trypan blue were made on the various suspensions using a Neubauer counting chamber. The number of cells counted for making the calculations was usually 100–150.

Immune peritoneal macrophages. Alloimmune peritoneal macrophages were collected from C57BL/Ks mice which had rejected an allograft of Sarcoma I (SaI) ascites tumour. The stock tumour was maintained by weekly passage in the A/Jax mouse.

More than 99 per cent of C57BL/Ks mice reject a standard inoculum of approximately 20×10^6 tumour cells within 8–10 days. On day 10 after the injection of tumour cells, the peritoneal cavity contains approximately 1×10^8 immune macrophages and very few tumour cells. 8 ml of SMEM lacking calf serum was injected into the peritoneum; the abdomen was massaged gently for a few seconds before the peritoneal washings were aspirated. Usually 5–6 ml of fluid could be recovered. The peritoneal washings of three or four mice were pooled, chilled to 4°, and manipulated in the cold to prevent cells from clumping and adhering to glass. The cell suspension was centrifuged for 4 minutes at 75*g* and the sedimented cells were resuspended in 20 ml of cold Eagle's minimal essential medium (MEM). Washing was repeated three times, and a standard suspension containing $2{\cdot}5 \times 10^6$ cell/ml was made by adding SMEM. The final preparations contained approximately 92–94 per cent macrophages.

Target cells. The L-cell, a permanent line of mouse fibroblasts, was used for the majority of experiments. Most of the H-2 antigens of the L-cell lacking in the C57BL/Ks mouse are present in the A/Jax mouse. Consequently the L-cell is specifically attacked by peritoneal macrophages from C57BL/Ks mice immune to A/Jax tissues.

Assay of target cell destruction. The *in vitro* plaquing technique of Granger and Weiser (1964) was used in which immune macrophages are superimposed at loci on a target cell monolayer. The technique scores plaques of clearing which result from the mutual death of interacting macrophages and target cells. 3×10^6 target cells were placed in a wide-mouthed 60/ml dilution bottle (monolayer area 18 cm^2) and cultured in 2·0 ml of SMEM at 37° in an atmosphere containing 5 per cent CO^2. The medium was replaced with

fresh medium 24 hours later. Two reaction loci were then established with a capillary pipette by allowing one drop of a suspension of immune peritoneal macrophages, containing $0{\cdot}5 \times 10^6$ cells, to fall on each of two selected sites on the monolayer of target cells. The plaquing bottles were not disturbed for 15 minutes in order to allow the immune cells to settle and adhere to the monolayer of target cells at the reaction loci. After 24 hours of incubation the medium was removed, the monolayers washed with physiological saline containing M/100 phosphate buffer, pH 7·2, (PBS), stained with crystal violet and examined for evidence of target cell destruction. The degree of clearing within plaques was determined and graded from − to +++. An increased density of staining, because of the large number of cells at the reaction locus, or lack of evidence of clearing was scored as −; less than 50 per cent clearing as +; greater than 50 per cent clearing as ++; and complete clearing as +++.

RESULTS

In order to determine whether membrane activity of interacting cells plays an important role in the killing of target cells, sodium fluoride (NaF) was used as an inhibitor of membrane activity. The concentrations of NaF employed have been reported to inhibit membrane activity selectively without interfering with protein synthesis (Cohn, 1966).

THE EFFECT OF SODIUM FLUORIDE TREATMENT ON THE CAPACITY OF IMMUNE PERITONEAL MACROPHAGES TO PRODUCE PLAQUES

Just prior to adding immune macrophages to target cell monolayers, the culture fluid was removed from the monolayers and 2·5 ml of 0·02 M NaF or 0·002 M NaF in physiological saline were added. Immune macrophages were then deposited at a locus on each monolayer. After standing 10 minutes at room temperature, the bottles were incubated for 30 minutes or 1 hour at 37°. The NaF solution was removed and the monolayers were washed with SMEM. The macrophages remained adherent to the monolayer. A control locus was then established on each monolayer with untreated immune macrophages. Incubation was continued for 24 hours and the degree of clearing in the plaques was estimated.

The results, presented in Table 1, show that plaque formation within 24 hours was markedly reduced by initial exposure of the interacting cells to 0·02 M or 0·002 M NaF for 30 minutes or 1 hour. Plaque formation at control loci produced with untreated immune macrophages was essentially unaffected. The wide variation in plaque formation resulted largely because the readings were taken early (at 24 hours) before plaque formation is complete, rather than at 48 hours. In subsequent experiments in which incubation was continued beyond 24 hours before readings were taken it was observed that plaques finally appeared in all cases at 48 hours. Thus initial treatment with NaF caused only a temporary block in the capacity of immune macrophages to produce plaques on target cell monolayers.

In additional experiments in which target cells were treated with 0·02 M or 0·002 M NaF for 1 hour and washed before adding immune macrophages plaque formation remained unaffected.

TABLE 1

PLAQUE FORMATION IN TARGET CELL MONOLAYERS, FOLLOWING SODIUM FLUORIDE (NaF) TREATMENT OF REACTION LOCI

Concentration of NaF	Period of NaF treatment	Degree of plaque clearing at 24 hours*	
		Treated loci	Untreated loci
0.02 M	First hour	− (7)†	
		+ (9)	+ (5)
		++ (2)	++ (10)
			+++ (3)
0.02 M	First 30 minutes	− (6)	++ (4)
			+++ (2)
0.002 M	First hour	− (3)	++ (1)
		+ (2)	+++ (5)

* − = increased density or no evidence of clearing at reaction locus,
\+ = less than 50 per cent clearing at reaction locus,
++ = more than 50 per cent clearing at reaction locus,
+++ = 'complete clearing' of reaction locus (>95 per cent).
† The numbers in parentheses designate the numbers of plaques.

TABLE 2

PLAQUE FORMATION IN TARGET CELL MONOLAYERS FOLLOWING 1 HOUR OF SODIUM FLUORIDE (NaF) TREATMENT, APPLIED AT VARIOUS INTERVALS AFTER THE ESTABLISHMENT OF REACTION LOCI

Concentration of NaF	Interval at which a 1 hour exposure to NaF was initiated	Degree of plaque clearing at 24 hours*	
		Treated loci	Untreated loci
0·02 M	0	− (4)†	
	1 hour	+ (1), ++ (3)	
	2 hour	++ (4)	
	4 hour	− (1), ++ (3)	
	(No exposure)		+ (1), ++ (3)
	(No exposure)		+++ (4)
0·002 M	0	+ (4)	
	1 hour	+ (1), ++ (2), +++ (1)	
	2 hour	+ (2), ++ (1)	
	4 hour	+ (1), ++ (2), +++ (1)	
	8 hour	+ (1), ++ (2), +++ (1)	
	(No exposure)		++ (4)

* − = increased density or no evidence of clearing at reaction locus,
\+ = less than 50 per cent clearing at reaction locus,
++ = more than 50 per cent clearing at reaction locus,
+++ = 'complete clearing' of reaction locus (>95 per cent).
† The numbers in parentheses designate the numbers of plaques.

KINETICS OF THE KILLING OF TARGET CELLS BY IMMUNE PERITONEAL MACROPHAGES

Irreversible changes leading to death of target cells apparently occur within a few hours after contact of immune cells with target cells (Granger and Weiser, 1964). The above results suggested that NaF could be used to examine the kinetics of the killing of target cells by immune macrophages.

Experiments were designed to determine how long the immune cell must interact with the target cell before irreversible cell damage occurs. Reaction loci were established and at subsequent intervals varying from 30 minutes to 4 hours the preparations were washed

with SMEM and exposed for 1 hour to 0·02 M or 0·002 M NaF before resuming culture in SMEM. The monolayers were stained and examined after 24 hours of incubation following NaF treatment.

The data in Table 2 show that the killing of target cells by immune macrophages was irreversibly initiated in large measure during the first hour of cell interaction.

THE EFFECT OF SODIUM FLUORIDE ON THE INCORPORATION OF [^{14}C]L-LEUCINE BY IMMUNE PERITONEAL MACROPHAGES

It has been reported that NaF in high concentrations causes an increase in the number of single ribosomes within the cytoplasm, presumably by blocking the formation of polysomes (LeBleu, Hurz, Burny and Marbaix, 1967). However, it did not seem likely that NaF in the low concentrations used above inhibited the immune activities of macrophages by a direct action on protein synthesis. This reasoning was based on the observation that the blockage of plaquing with chloramphenicol required at least 2 hours of preincubation with the agent and its continued presence in the culture medium (Granger and Weiser, 1966). To test this thesis the influence of NaF on the capacity of cultured immune macrophages to incorporate [^{14}C]L-leucine was studied.

2 ml of a standard suspension of washed immune macrophages in SMEM were placed in 60-ml dilution bottles and allowed to stand for 1 hour at 37°. The medium was removed from each resulting macrophage monolayer and 2 ml of a solution containing isotope (0·05 μg/ml sp. act. 248 mCi/m-mole) and 0·02 or 0·002 M NaF were added. At intervals sample bottles were assayed for isotope. Controls included untreated macrophage monolayers and macrophage monolayers treated with puromycin, a known inhibitor of protein synthesis. Untreated macrophages in the original suspension were tested for their ability to produce plaques in monolayers of target cells. At various intervals the total incorporation of [^{14}C]L-leucine (counts/min) into TCA-precipitable material was determined in a Beckman liquid scintillation counter. The results presented in Fig. 1 show that puromycin (100 μg/ml) blocked the incorporation of label immediately. The shut-down of incorporation of label was delayed for 30 minutes with 0·02 M NaF, whereas 0·002 M NaF had no effect on the incorporation of label, even though it retards plaque formation.

DISCUSSION

The present investigations indicate that the action of NaF in inhibiting plaque development resides solely in its capacity to block membrane activity of the immune macrophage and not the target cell.

Previously Granger and Weiser (1966) demonstrated that plaque formation was inhibited when immune cells were treated with the metabolic inhibitors actinomycin D and chloramphenicol but not when target cells were given the same treatment. The capacity of immune macrophages to destroy target cells was abolished only when immune cells were incubated with inhibitor for at least 2 hours before adding them to target cells.

In the present work, using the selective membrane inhibitor NaF, it was observed that pre-incubation of immune macrophages with NaF was not required for blocking the formation of plaques and that the concentration of NaF sufficient to block plaquing did not affect protein synthesis by immune cells. Sodium fluoride probably prevents target cell killing by interfering with plasma membrane activity of immune macrophages.

The block of plaque formation previously effected with actinomycin D and chloramphenicol could have resulted from inhibition of membrane activity secondary to inhibition of nucleic acid or protein synthesis. The 2 hours of pre-incubation of immune cells with actinomycin D or chloramphenicol required to block plaque formation may reflect the time needed to deplete the pools of precursors required for membrane turnover.

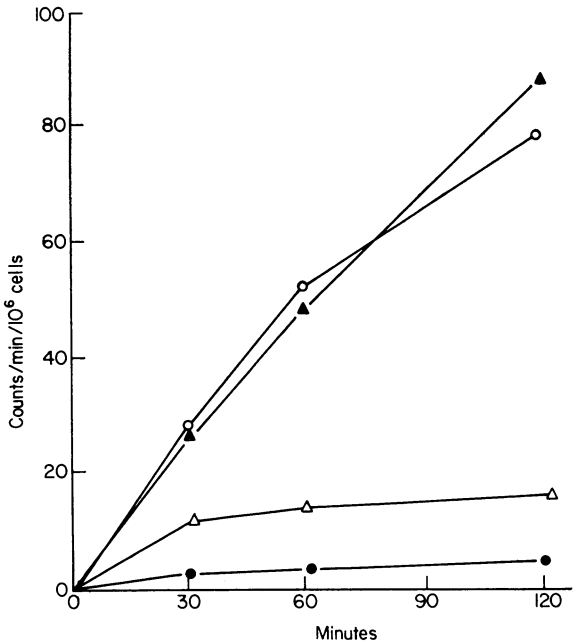

FIG. 1. The incorporation of [^{14}C]L-leucine by alloimmune macrophages following treatment with Puromycin and sodium fluoride (NaF). ●, Puromycin; △, 0·02 M NaF; ▲, 0·002 M NaF; ○, untreated.

Earlier results (Weiser *et al.*, 1969) showed that within 2 hours a substance is released into the supernatant from interacting immune macrophages and target cells which inhibits the growth of specific target L-cells. Granger (1965) obtained cytological evidence of target cell injury after 3½ hours of contact with immune macrophages. It is probable that these two previous observations are related to the present finding that irreversible killing of target cells is initiated to a large extent within the first hour following contact with immune cells.

Obviously a metabolically active immune macrophage, but not target cell, is essential for target cell killing. In addition, the present results with NaF indicate that the capacity of immune macrophages to kill target cells demands membrane activity which may be concerned with the release or secretion of a cytotoxin.

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