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Studies on the Effect of Macrophages in an *in vitro* Graft Reaction System

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Summary. A study was made of the immunospecific lysis of mouse fibroblasts by rat lymphocytes sensitized *in vitro* against mouse cells in the presence or absence of macrophages. The cytolytic activity of rat lymph node cells decreased if they were filtered through a glass bead column prior to sensitization. This decrease could be reversed by the addition of defined amounts of peritoneal macrophages. The ratio of added macrophages to lymphoid cells was found to be critical. While small amounts of macrophages added within a defined dose range caused enhancement of activity, both of glass bead treated and of untreated populations, addition of higher quantities resulted in the inhibition of the reaction. A possible regulating role of macrophages in immune reactions is suggested.

INTRODUCTION

Macrophages have been demonstrated to play a triggering role in antibody production to a number of antigens (Fishman, 1961; Askonas and Rhodes, 1965; Gallily and Feldman, 1967; Mitchison, 1969). The question whether macrophages participate in any way in the induction of cell mediated immunity remained open. Blastogenesis of lymphocytes triggered either by bacterial products or in mixed leucocyte reactions was claimed to depend on the presence of macrophages (Hersh and Harris, 1968; Cline and Swett, 1968; Gordon, 1968). The blastogenic response might reflect a stage in the immune sensitization of lymphocytes, yet its precise nature with regard to the complete cell-mediated immune reaction is not quite clear. Therefore, the significance of these observations with regard to the immune response *per se* requires further investigation using systems where both the sensitization and the effector phase could be experimentally induced. The *in vitro* system for the induction of a primary graft reaction developed in our laboratory seemed therefore suitable for such an analysis (Ginsburg and Sachs, 1965; Berke, Ax, Ginsburg and Feldman, 1969). It is based on sensitizing rat lymphocytes on monolayers of mouse fibroblasts. The sensitizing rat lymphocytes undergo blastogenesis, replicate and after 5–6 days of culture acquire the capacity to lyse mouse fibroblasts genetically identical with the sensitizing mouse cells. Lysis is measured following transfer of the sensitized lymphocytes to ^{51}Cr-labelled test monolayers, by determining the release of the radiochromium. Our previous studies indicated that this *in vitro* induced cellular immune reaction involves the co-operation between thymus and bone marrow-derived lymphocytes, both of which seem to recognize the mouse antigens (Lonai and Feldman,

1970, 1971). In the present study we aimed at testing whether macrophages play a role in this graft reaction.

MATERIALS AND METHODS

Animals. Lewis/Mai female rats, 2½–3 months of age (Microbiological Associates Bethesda, Md.) were used as donors of lymph node cells. Embryos of C3HeB mice from the Weizmann Institute Animal Breeding Center were used to prepare monolayers of mouse embryo fibroblasts.

The in vitro *graft reaction system.* The technique used was described in detail previously (Berke *et al.*, 1969; Lonai and Feldman, 1970). We give here only a brief outline of the system. Pooled rat lymph node cells are plated on X-irradiated C3H embryo fibroblast monolayers in 60 mm diameter Falcon Petri dishes (Falcon Plastics, Los Angeles), 20–30 $\times 10^6$ cells per dish. The cells are cultured for 5 days in Dulbecco's modified Eagle's medium (EM) containing 20 per cent horse serum in a 37° incubator with 7–10 per cent CO_2 + air supply, 100 per cent humidity. After 5 days the lymphoid cells are collected, counted and the viability is determined by dye exclusion test. Two and a half million viable lymphoid cells are transferred to X-irradiated C3H fibroblast test monolayers (0·6–0·8 $\times 10^6$ fibroblasts) prelabelled with ^{51}Cr in 35 mm diameter Falcon Petri dishes. These are incubated for an additional 20 hours after which the chromium release is determined by measuring separately the activity of the supernatant medium, and after trypsin treatment, that of the residual fibroblasts. The activity of the culture is defined as the per cent radioactivity released on the basis of the total activity of the culture, after subtracting the spontaneous ^{51}Cr release determined by incubating labelled monolayers in the absence of lymphoid cells.

Production of peritoneal exudate cells (PEC). Lewis rats were injected intraperitoneally with 5 ml of aged thyoglycollate medium (Difco Laboratories, Detroit). After 4 days the animals were killed and the peritoneal cavity was washed twice with 25 ml sterile phosphate buffered saline (PBS). The cells were washed and resuspended in EM containing 20 per cent horse serum. As seen on Giemsa-stained slides, the peritoneal exudate contained 60–75 per cent large mononuclear cells, which we refer to as macrophages.

The purification of peritoneal exudate cells (PEC). The collagen-collagenase method of Gallily and Feldman (1967) was used; 10^7 PEC were seeded in Hanks's medium plus 20 per cent horse serum in 60 mm Petri dishes overlayed with rat tail collagen. The cells were cultured for 1 day after which the plates were rinsed with Hanks's medium containing 0·05 per cent collagenase (bacterial collagenase, grade B, Calbiochem, Los Angeles). Incubation with the enzyme was carried out for 30 minutes on a rotary shaker at 37°. The collagen digest resulted in 30–60 per cent yield of the plated macrophages and the cell suspensions contained more than 95 per cent macrophages.

Glass bead filtration. Glass columns (2·4 × 35 cm) were filled with glass beads of about 12–14 mesh (Arthur Thomas Co., Philadelphia, No. 4285 M20). The inner volume of the column was 75 ml. The columns were set up in a 37° room and they were filled with warm EM + 20 per cent horse serum. Lymph node cells, 10^9, were layered on the column and were allowed to soak in slowly by opening the flow. After 30 minutes of incubation the column was washed with warm medium at a flow rate of about 5 ml per minute. The first 50 ml did not contain cells and were discarded. The cells not bound were eluted with further 60 ml medium which was collected in 2 or 3 fractions. The elution yielded about

50 per cent of the originally applied cells. In certain cases, in an attempt to release bound macrophages, the column was washed with 0·02 per cent Na-EDTA in which case the EM applied was devoid of Ca^{++} and Mg^{++}, or with 0·3 per cent trypsin solution. The fractions were washed, the cells were counted and used for culturing.

RESULTS

THE EFFECT OF GLASS BEAD FILTRATION ON THE CELL COMPOSITION OF LYMPH NODE SUSPENSIONS

Before starting experiments aimed at studying the effect of depletion or addition of macrophages on the *in vitro* graft reaction, we estimated the macrophage-content of rat lymph nodes used for culturing. Concomitantly we studied the effect of glass bead filtration on the macrophage content of the suspension and the possibility of eluting cells from the glass bead column with EDTA or trypsin treatment.

TABLE 1

THE EFFECT OF GLASS BEAD FILTRATION ON THE MACROPHAGE CONTENT OF LYMPH NODE SUSPENSIONS*

	Per cent yield	Per cent lymphocytes	Per cent macrophages	Macrophage change Per cent of control
Control	—	91·0	0·6	—
Filtered	42	87·8	0·25	42
EDTA	7	80·2	4·1	682
Trypsin	7	87·9	0·8	132

* Average values of four experiments.

Rat lymph node cell suspensions were applied to the glass bead column. The effluent was collected and the cells adhering to the column were treated either with Na-EDTA or with 0·3 per cent trypsin. The eluted fractions were centrifuged, washed and smears were prepared and stained for the determination of cell types. In four such experiments it was found that the glass bead column retains about 40–50 per cent of the cells applied and that the EDTA or trypsin treatment yielded an additional 7–8 per cent cells. Ninety-five per cent of the cells from all fractions were viable. It was found that suspensions of rat lymph node cells contain 0·5–0·8 per cent macrophages. This quantity decreased to 0·15–0·30 per cent after filtration through the glass bead column. The EDTA treatment of the residual cells on the column resulted in an effluent containing 6–7 times more macrophages than in the original lymph node cell suspension. These data (Table 1) demonstrate that the glass bead filtration method only decreases but does not eliminate the normally low amount of macrophages in a lymph node cell suspension. The EDTA treatment of the retained cells, on the other hand, results in a cell suspension which contains 6–7 times more macrophages than the untreated suspension, but still has an overwhelming majority of lymphocytes.

THE EFFECT OF GLASS BEAD FILTRATION ON THE *in vitro* ACTIVITY OF LYMPH NODE CELLS

Lymph node cell suspensions were filtered through a glass bead column and the effluent cells were tested in the *in vitro* graft reaction system. Twenty million lymphocytes

were plated per mouse monolayer culture and were incubated for 5 days, after which the sensitized cells were transferred to ^{51}Cr-labelled test monolayers for the determination of the lytic capacity. It was found that the glass bead effluent had regularly manifested a reduced lytic activity (Table 2). The extent of reduction, however, was not constant and in several cases the filtered cells had almost the same activity as the control. The highest inhibition was obtained with cells which were eluted by the first 20 ml of the cell-containing effluent (Table 2, experiments 1 and 2). Therefore, in subsequent experiments only this fraction was used as the glass bead-treated fraction. When macrophage-rich cell suspensions were added to the filtered fraction prior to plating (Table 2, experiment 3 and 4) an elevation of the activity was observed, when either small amounts of peritoneal exudate cells or EDTA effluent were added to the filtered fraction.

TABLE 2

THE EFFECT OF GLASS BEAD FILTRATION ON THE *in vitro* ACTIVITY OF LYMPH NODE CELLS. RECONSTITUTION WITH EDTA EFFLUENT OR PERITONEAL EXUDATE CELLS

	Per cent lysis ± S.E.*	Per cent of control
1. Control	40·4 ± 0·8	100·0
Filtered a	9·5 ± 0·8	23·5
Filtered b	20·0 ± 3·7	49·5
2. Control	43·8 ± 4·2	100·0
Filtered a	12·0 ± 2·1	27·4
Filtered b	19·5 ± 1·3	44·5
Filtered c	31·7 ± 3·2	72·4
EDTA effluent	23·3 ± 1·0	53·2
3. Control	60·7 ± 3·6	100·0
Filtered	25·4 ± 0·9	41·8
Filtered + EDTA effluent 1:1	35·6 ± 2·5	58·6
4. Control	46·3 ± 0·6	100·0
Filtered	39·7 ± 1·4	85·7
Filtered + 2×10^6 PEC	2·8 ± 1·2	6·0
Filtered + 2×10^5 PEC	45·9 ± 1·7	99·1

* S.E.—standard error of three or four individual cultures.

The fact that the lytic activity developing in these cultures during sensitization on the foreign monolayer decreases if the quantity of glass bead-adherent cells is reduced, suggested that the macrophages have a certain role in this cell-mediated immune reaction. This was supported by our finding that the addition of small amounts of macrophages either in the form of 1 per cent PEC in the lymphoid suspension, or in the form of the EDTA effluent enriched in macrophages, has a reconstitutive effect on the lytic activity of the treated cultures (Table 2, experiments 3 and 4). The addition of EDTA effluent cells (containing 3–5 per cent macrophages) to glass bead filtered lymph node cells in a ratio of 1:1 resulted in an increased activity (Table 2, experiment 3), but if this macrophage-enriched fraction was plated alone, its activity was about 30 per cent less than that of the untreated control (Table 2, experiment 2). If filtered lymph node cells were complemented with 2×10^6 PEC (10 per cent of the lymphocyte population) the culture lost about 90 per cent of its activity, although an amount of 2×10^5 cells from the same suspension caused 20 per cent enhancement (Table 1, experiment 4). It thus appears that at certain cell concentrations macrophages increase the activity of the system, and at higher concentration they inhibit it. This dual effect of macrophages on the *in vitro* reaction in culture was studied in subsequent experiments.

THE EFFECT OF DIFFERENT AMOUNTS OF MACROPHAGES ON THE *in vitro* GRAFT REACTION

Lymph node cell suspensions were filtered through glass bead columns. Treated and untreated cells were plated on fibroblast monolayers. 2×10^4–1×10^6 collagen purified macrophages were added to 20×10^6 glass bead filtered cells (0·1 per cent–5 per cent) immediately after plating. After 5 days the lytic effect was measured. The results (Fig. 1) indicated the dual effect observed formerly (Table 2). Addition of 2×10^4 and 5×10^4 macrophages resulted in low levels of enhancement of the lytic reaction, compared to the level of glass bead filtered cells, whereas 10^5 or 2×10^5 (0·5–1 per cent) macrophages strongly increased the activity, surpassing even the effect of untreated cells. When 10^6

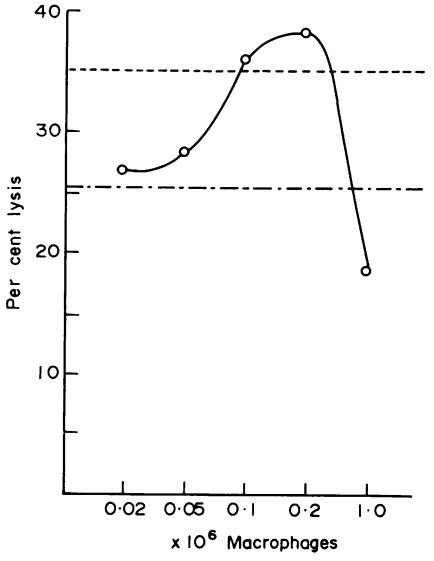

FIG 1. *In vitro* lytic effect of lymphocytes filtered on glass bead columns. Reconstitution with purified macrophages. - - -, untreated control: - . -, filtered on glass bead column; ○ - ○, effect of addition of macrophages.

macrophages (5 per cent) were added to the cultures a significant decrease in lytic activity was observed. Thus, the depletion of macrophages from a lymph node cell suspension decreases the lytic activity of the *in vitro* graft reaction. This impairment can be reversed by adding a certain amount of purified macrophages. Higher concentrations of macrophages have an inhibitory effect.

In the next experiment we tested whether macrophages will affect untreated lymph node cell suspensions in a manner similar to that observed with macrophage depleted lymphoid population. Lymph node cells were plated on fibroblast monolayers and the cultures were immediately supplemented with different amounts of PEC. Cultures consisting of 20×10^6 untreated lymphocytes or of the same amount of PEC were processed as control. The results were similar to those obtained following addition of macrophages to filtered lymph node cell populations (Fig. 2). High amounts (5–10 per cent) of macrophages inhibited the lytic activity, and in this case also, there were certain quantities of

macrophages which had a slight enhancing effect on the untreated lymph node suspensions. Cultures containing PEC cells only had no activity.

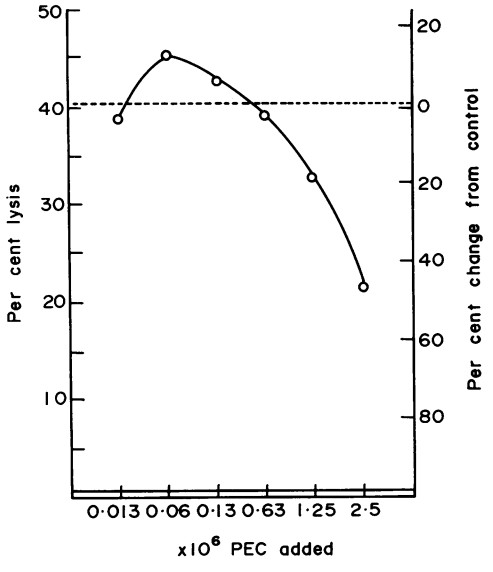

FIG. 2. The effect of addition of PEC to untreated lymph node cells. - - -, the activity of untreated lymphocytes; —, the activity of PEC alone; O - O, the effect of addition of PEC.

DISCUSSION

Our results demonstrate that cell suspensions of rat lymph nodes deficient in glass-adhering cells have a decreased capacity to lyse mouse fibroblasts following *in vitro* sensitization. This decreased activity could be restored by adding certain amounts of peritoneal exudate cells or purified macrophages. The degree of impairment due to glass bead filtration was not constant throughout the experiments. This inconsistency might be explained by the fact that macrophages constituting a very small fraction of the lymph node cell suspension are not eliminated completely by the glass bead filtration, as was observed by cytological examination. The amounts of macrophages needed to restore the deficient activity of filtered cells was around 1 per cent of the original cell population. Hence the immune lysis of mouse cells by sensitized lymphocytes depends on a relatively low amount of macrophages. We have recently demonstrated that the *in vitro* graft reaction is based on a bicellular lymphoid interaction (Lonai and Feldman, 1970, 1971). The present findings concerning the requirement for macrophages falls in line with the findings of Mosier and Coppleson (1968) who demonstrated a necessary interaction between macrophages and two types of presumably lymphoid cells in an *in vitro* antisheep red blood cell system. It thus appears that both humoral and cellular immune reactions might involve co-operation of three types of cells: two different types of lymphocytes and the macrophages.

A striking feature of our findings is that macrophages have reconstituting or enhancing effect only within a very distinct dose range. Above this range they produce a well marked

inhibition. The inhibitory effect was observed both with cell populations depleted of glass bead adherent cells and with normal lymph node cell suspensions.

The inhibitory effect of large quantities of macrophages with concurrent stimulation exerted by well defined smaller amounts of macrophages has been observed in several different immunological systems. The dose dependence of inhibition or stimulation exerted by macrophages was first described by Harris (1965) who measured the antigen-induced DNA synthesis of spleen cells from rabbits immunized with bovine serum globulin. Hersh and Harris (1968) and Cline and Swett (1968) report basically similar observations when they introduced different amounts of macrophages to lymphocyte cultures stimulated by bacterial antigens. Similar observations were made on young mice *in vivo* by Argyris (1968). More recently Hoffman (1970) demonstrated dose response curves for added macrophages, strikingly similar to ours, in an *in vitro* primary anti-sheep red blood cell system. These data suggest that macrophages might act as regulators of the multiplication and/or differentiation of the immunocompetent cells. Similar ideas were expressed by Harris in 1965 with regard to antibody production. Whether the suggested regulatory function of macrophages is independent of the processing function, or whether the two apparent phenomena are manifestations of one essential process, remains an open question.

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