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The Mechanism of Tumour Cell Induced Inhibition of Human Leucocyte Migration*

CATHERINE E. ROSS, ALISTAIR J. COCHRAN,† ALAN M. JACKSON, RONA M. MACKIE and LINDSAY J. OGG

University of Glasgow Departments of Pathology and Dermatology, The Western Infirmary, Glasgow, United Kingdom

Abstract—*We investigated the mechanism of leucocyte migration inhibition by formalin-fixed tumour cells. Peripheral blood leucocytes were separated into the component populations; granulocytes, lymphocytes, lymphocyte subpopulations and monocytes and incubated for* 24 hr *with formalinised melanoma cells and formalinised control cells. The supernatants of these cultures were tested for their capacity to inhibit the migration* in vitro *of normal peripheral blood leucocytes. Inhibitory activity was generated by cocultures of melanoma cells with populations containing a substantial proportion of lymphocytes. Studies with populations enriched for T lymphocytes indicated that T cells generated the inhibitory activity although our inability totally to purify the T cells meant that a (cooperative) role for Fc receptor bearing lymphocytes including B cells could not be excluded.*

INTRODUCTION

Most studies of the leucocyte migration inhibition technique (LMIT) in cancer patients, including our own early reports [1–3] describe the use of soluble or microparticulate cell extracts as antigen. Dissatisfaction with the heterogeneity of this type of material, lack of consistent dose–responses, ignorance of the nature of the active components and practical difficulties in providing quantities of "purified" [4] tumour-associated antigens (TAA) sufficient for multiple tests lead us to introduce formalin-fixed single cell suspensions as "antigens" in the LMIT [5]. This procedure is sufficiently different that we felt compelled to analyse the mechanism of leucocyte migration inhibition by formalinised cells.

Although leucocyte migration inhibition can be mediated by soluble products of lymphocytes [6–8] it has been reported that migration may be inhibited by a direct effect of antigen on polymorphonuclear cells [9, 10]. With whole tumour cells as "antigen" mechanical impedence by, for instance, immune adherence might also cause migration inhibition.

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†Correspondence to: Dr. A. J. Cochran, Pathology Department, Western Infirmary, Glasgow, G11 6NT.

MATERIALS AND METHODS

Venous blood was obtained from 10 patients with malignant melanoma and 10 disease free individuals, heparinised and fractionated.

1. *Whole leucocyte preparation*

After gravity sedimentation the white cell rich plasma was removed and spun at 400 ***g*** for 10 mins at 22°C. The cells were washed twice in phosphate buffered saline (PBS) and resuspended to a concentration of 2.5×10^6/ml in Eagles' minimum essential medium (MEM) supplemented with 10% foetal calf serum (MEMF).

2. *Ficoll-Hypaque preparations*

Ten millilitres heparinised blood were layered onto 10 ml Ficoll-Hypaque (FH) solution (Pharmacia, Sweden) and spun at 400 ***g*** for 20 min at 22°C. The cells at the plasma/FH interface were pipetted off, washed twice in PBS for 5 min at 400 ***g*** and resuspended as in (1) above.

3. *"Purified" lymphocyte preparation*

Cells obtained by method 2 were incubated in plastic Petri dishes (60 × 15 mm) for 2 hr at 37°C. The non-adherent cells were washed off gently with MEM.

4. *Adherent cell preparation*

These were removed from the base of Petri dishes by gentle scraping with a rubber policeman.

5. *Granulocyte preparation*

The cells which had spun through FH were resuspended in the donor's plasma, and 2 ml of 6% Dextran added. After the red blood cells had sedimented the granulocyte-rich fraction was removed and treated as in (1) above.

6. *Lymphocyte populations depleted of cells bearing Fc receptors*

Preparation of EA rosettes. Heparinised blood was separated over FH and the plasma retained. The lymphocyte fraction was washed twice in PBS for 5 min at 400 ***g*** and resuspended in MEMF to a concentration of 4 × 10^6 cells/ml.

Half a millilitre aliquots of cell suspension were added to 0.5 ml of a 1% (v/v) suspension of chicken erythrocytes which had been sensitized with rabbit IgG anti-chicken erythrocyte (EA) (1/500) in 3 × 3/8 in. glass tubes [11]. The tubes were spun at 200 ***g*** for 5 min at 22°C, the supernatant removed and the cell pellet resuspended in 4 vol of autologous plasma.

Separation of EA rosettes. Four millilitres of plasma containing EA rosetting cells were layered onto 3 ml of Ficoll-Metrizoate (Nyegaard, Oslo) of specific gravity 1.085 in 4 × 1/2 in. tubes. These were spun at 400 ***g*** for 30 min. The band of lymphocytes which had not formed rosettes was removed, resuspended in an equal volume of Eagles' medium and spun at 400 ***g*** for 30 min. These cells were washed twice at 200 ***g*** for 5 min, counted and resuspended to 4 × 10^6/ml.

Fixation of rosettes. The rosette containing pellet was resuspended in 0.5 ml of 3% glutaraldehyde for 20 min. The tubes were then topped up with water and spun (400 ***g***). The supernatant was removed and the cells were resuspended in 0.25 ml trypan blue (0.75%). Two slides were prepared from each tube for counting of rosettes.

7. *Differential leucocyte counts*

Film spreads from all leucocyte fractions in this study were stained by May Grunwald–Giemsa and differential counts performed.

8. *Two-stage leucocyte migration inhibition tests*

Two millilitre cultures (5 × 10^6 cells) of leucocytes with and without formalinised cells at 50:1 and 100:1 (leucocytes:FC) were established in screw-topped, round bottomed glass tubes (100 × 9 mm). Cultures were incubated for 24 hr at 37°C after which the centrifuged (400 ***g*** × 10 min) supernatants were removed and immediately tested.

Indicator cells were prepared from control blood (method 1) and resuspended in MEMF to 1 × 10^8 cells/ml. For each supernatant four capillaries were filled with this suspension, sealed at one end with clay and spun at 200 ***g*** for 5 min. The resulting cell buttons were separated with a diamond and mounted horizontally on the base of disposable tissue culture plates which were filled with the appropriate culture supernatant, closed with a coverslip and incubated at 37°C for 18–24 hr. Areas of cell migration were drawn and measured by planimetry.

The migration index was calculated by dividing the mean area of migration of test leucocytes (derived from four separate capillaries) in the supernatant of 24 hr cultures of leucocytes and FC antigen by the mean area of migration of indicator leucocytes from the same donor in the supernatants of 24 hr cultures of test leucocytes without antigen.

Significance at the 5% level was assessed by the Mann–Whitney Wilcoxon U-test of ranking.

RESULTS

Differential cell counts

The proportion of granulocytes to mononuclear cells was radically altered by the various manipulations but no fraction was completely free of any one cell type (Table 1).

The technique which was least satisfactory was the adherent cell method which yielded a major increase in the proportion of monocytes but permitted a disappointingly high number of lymphocytes to remain. In retrospect this may have been due to insufficiently vigorous

Table 1. *Differential leucocyte counts of cell preparations used*

Cell preparation	No.	Granulocytes Mean ± S.E.M.*	Lymphocytes Mean ± S.E.M.	Monocytes Mean ± S.E.M.
Whole leucocyte	8	61† ± 3.6	32 ± 3.6	7 ± 2.0
Ficoll-Hypaque	8	15 ± 3.7	82 ± 3.2	3 ± 0.9
"Purified" lymphocyte	8	3 ± 1.6	92 ± 2.7	5 ± 1.5
Adherent cells	8‡	2 ± 1.8	28 ± 3.7	70 ± 3.7
Granulocytes	8	94 ± 2.0	4 ± 1.7	2 ± 0.6

*Standard error of the mean.
†Figures are percentages.
‡These figures are less exact than for other preparations as the removed adherent cells were readily destroyed by the mechanical spreading employed during film preparation and some showed as partly damaged and therefore less confidently identifiable cells.

washing of the dish following incubation and prior to harvesting.

EA rosette counts

The percentages of EA rosette forming (Fc receptor positive) cells were considerably reduced by the techniques employed, the mean before depletion being 25% and after 4.5%. However, we were unable completely to eliminate these cells (Table 2).

any of the ten control donors' cell preparations cocultured with FMC.

Further controls included MEMF conditioned by the presence of the whole leucocyte preparations alone, FH preparations alone, "purified" lymphocytes alone, adherent cells alone, granulocytes alone and FMC or FCC alone, and MEMF conditioned by incubation at 37°C for 24 hr in the absence of leucocytes and FC. No inhibitions were obtained using the supernatants from FC alone, MEMF alone, leucocytes, adherent cell preparations or granulocyte preparations alone. However, supernatants from 3 patients' FH preparations and from 1 patients' "purified" lymphocyte preparation, cultured alone, without added FC, were inhibitory.

Table 2. *Effect of depletion of cells bearing Fc receptors on % EA rosetting cells in Ficoll-Hypaque separated lymphocytes*

Preparation	% EA Rosettes Before removal Fc receptor bearing cells	After removal Fc receptor bearing cells
1	22	6
2	24	2
3	23	7
4	29	3

Leucocyte migration inhibition by the supernatants of the different cell preparations incubated with FTC

The activity of the supernatants from coculture of the melanoma patients' different leucocyte preparations with FMC varied widely (Table 3). FMC/whole leucocyte supernatants were inhibitory in only 3/10 experiments, whereas the FMC/FH preparation supernatants and the FMC/"purified" lymphocyte supernatants were inhibitory in 8/10 and 3/6 experiments respectively. Supernatants from FMC/adherent cell cultures were not inhibitory and supernatants of FMC/granulocyte cultures inhibited in only 2/8 experiments. *No inhibitions were obtained using the supernatants from*

FH preparations from melanoma patients and control donors were cultured with non-melanomatous FTC (ovarian carcinoma, renal carcinoma). Supernatants from 2/24 melanoma FH preparations and 2/17 control donor FH preparations cocultured with non-melanoma tumour cells inhibited leucocyte migration.

In 4 experiments the activity of the supernatants of FMC/FH cultures was compared

Table 3. The effect of supernatants from the coculture of the various leucocyte preparations and FC on the migration of normal leucocytes

Leucocyte donors	Type of tumour cells used as "antigen"	Whole leucocytes Positive/tested	Ficoll-Hypaque Positive/tested	"Purified" lymphocytes Positive/tested	Adherent cells Positive/tested	Granulocytes Positive/tested
Melanoma patients	Melanoma (FMC)	3/10	8/10	3/6	0/5	2/8
Melanoma patients	Other tumours* (FCC)	NT	2/24	NT	NT	NT
Control donors	Melanoma	0/10	0/10	0/7	0/5	0/8
Control donors	Other tumours	NT	2/17	NT	NT	NT

*Ovarian carcinoma and renal carcinoma.
NT = not tested.

with that of supernatants from cultures of FMC and lymphocytes from the same individuals depleted of EA rosette-forming cells (Table 4). Significant migration inhibition was obtained with both lymphocyte preparations in 1/4 cases and with the EA rosette forming cell depleted preparation only in 2/4 tests. One test was negative in both cases.

DISCUSSION

The migration of melanoma patients' leucocytes is inhibited preferentially by FMC [5]. The experiments described in this paper indicate that such inhibitions are, at least in part, due to materials produced by melanoma patients' leucocytes in contact with FMC. Most reactive were the FH populations (8/10) and the "purified" lymphocyte population (3/6), which contained the highest proportions of lymphocytes. The adherent cell fraction was the only one in which there was no reactivity in leucocytes derived from melanoma patients. Although these preparations contain lymphocytes non-reactivity may indicate that the relatively adherent population of lymphocytes present is not lymphokine productive. These data suggest strongly that the cells producing the inhibitory materials are lymphocytes. Their occurrence in melanoma patients and selective responsiveness to FMC suggests that they are specifically sensitised to TAA on melanoma cells.

In further experiments we attempted to investigate the class(es) of lymphocytes involved. Since delayed hypersensitivity reactions are regarded as being mediated by T lymphocytes it was initially assumed that antigen-induced production of migration inhibitory factor would also be a function of T lymphocytes. However there have been recent claims that under some circumstances non-T lymphocytes can produce lymphokines [8, 12].

In an attempt to ascertain whether T lymphocytes were active in the system under examination we removed EA rosetting cells (Fc-receptor bearing cells) by spinning out EA rosetted cells over Ficoll-Metrizoate, a procedure which left only 5% of lymphocytes capable of forming EA rosettes when the residual lymphocyte population was retested. Inhibitions were obtained using supernatants of cultures of EA rosetting cell-depleted fractions and FMC, indicating that lymphocyte populations in which T cells predominate are certainly capable of producing the inhibitory factor. This view is supported by the

Table 4. Comparing the effect of supernatants of cultures of FMC with Ficoll-Hypaque preparations and lymphocyte preparations depleted of EA rosette-forming cells

Preparation	Ficoll-Hypaque preparation migration index	EA-rosetting cell depleted preparation migration index
1	0.80*	0.73*
2	0.98	0.82*
3	0.85	0.79*
4	0.95	0.91

*Migration inhibition significant at the 5% level (Mann–Whitney Wilcoxon U-test).

observations of Fimmel [13] who depleted lymphocyte suspensions of E rosette-forming cells and found the capacity to produce leucocyte inhibitory activity to be lost in depleted populations. That removal of EA rosetting cells rendered the residual cell population reactive with FMC in two instances raises the possibility that in the combined cell populations suppressor cells may be active.

The cells removed by EA rosetting are heterogeneous, comprising IgG Fc receptor bearing B lymphocytes, monocytes, K cells, granulocytes and probably a minority of T cells. As a prelude to investigating the capacity of these classes of cells in lymphokine production we attempted the selective removal of T cells. To the present we have been unable to recover sufficient non-T cells after removal of E rosetting lymphocytes to allow cultures to be set up. We thus have no information on the ability of B lymphocytes, K cells or Fc+ve T lymphocytes to produce or cooperate in the production of migration inhibitory activity. The simple fractionation experiments employing FH separation and adherence to plastic suggest that granulocytes and monocytes do not produce material of this kind. Other workers have suggested that both T and B lymphocytes may be able to produce MIF when stimulated by specific antigen [14]. In guinea-pig studies, however, Yoshida *et al.* [12] showed that sensitised T cells were able to produce MIF in response to specific antigen, and that B cells only produced MIF in response to mitogenic antigens such as PPD. Landolfo *et al.* [15] have shown that sensitised T lymphocytes respond to even allogeneic intact tumour cells bearing the sensitising antigen by producing MIF *without* the assistance of histocompatible macrophages, in contrast to the response to soluble tumour antigens which requires the presence of histocompatible macrophages. Our data confirm responsiveness to allogeneic tumour cells but as our lymphocyte populations all contained some macrophages, we cannot draw any conclusions on the need for macrophage cooperation in our studies. It is our intension to examine this important point by the use of more extensively purified preparations.

Leucocyte migration inhibition by formalinised tumour cells is thus caused at least in part by inhibitory substances produced by lymphocytes. The two-stage technique is simple to perform; and the specificity shown in comparisons of melanoma patients and control donors suggests its possible utility for the detection of cell-mediated immunity to tumour associated antigens in cancer patients.

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