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Surface antigens of the murine cytostatic peritoneal macrophage

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Summary. By rosetting techniques, peritoneal exudate cells (PEC) from mice stimulated intraperitoneally with starch have been shown to be a mixed population of cells consisting of the following subpopulations of cells: 75% FcR, 26% C3R, 18% Ig^+, 29% Ia^+, Ig^- and 5% Thy-1^+, Ig^-. By separating rosetting from non-rosetting cells, it was possible to establish the phenotype of the PEC which was cytostatic for tumour cells. This cell possessed receptors for Fc and C3 but lacked surface Ig, the Thy-1 antigen and I-region controlled antigens and was NSE positive. Thus by presently available criteria, the cytostatic PEC can be identified as a macrophage. The lack of Ia distinguishes this type of macrophage from the antigen-presenting macrophage which bears Ia.

INTRODUCTION

Elicited murine peritoneal exudate cells (PEC) are cytostatic and often cytolytic for tumour cells and virus infected cells (Hibbs, 1976; Keller, 1976; Goldman & Hogg, 1977). In general, these PEC are assumed to be macrophages (Mph) on the basis of phagocytosis or adherence to plastic but in some activation regimes, such as BCG treatment, high levels of cytolytic natural killer or NK cells are generated (Tracey, Wolfe, Durdile & Henney, 1977; Wolfe, Tracey & Henney, 1977). There is little known about the antigenic heterogeneity of Mph performing different functions. Cytostatic Mph may belong to a restricted subset of cells possessing a particular selection of surface antigens or they may be antigenically heterogenous. Most Mph from the earliest identified monoblast to the mature Mph have Fc receptors (FcR) (Goud, Schotte & van Furth, 1975) which bind IgG2a and IgG2b (Diamond, Bloom & Scharff, 1978) but only the more mature cells are phagocytic and possess receptors for complement (C3R) (Goud *et al.*, 1975). In addition, a proportion of Mph bear *I* region associated antigens (Ia) (Cowing, Schwartz & Dickler, 1978) and these Mph process and present antigen to T cells (Shevach & Rosenthal, 1973; Schwartz & Paul, 1976; Erb, Feldmann & Hogg, 1976; Cowing, Pincus, Sachs & Dickler, 1978; Ahmann, Sachs & Hodes, 1978; Hodes, Ahmann, Hathcock, Dickler & Singer, 1978). but it is not known whether this type of macrophage assisting in initiating an immune response, also has the capacity to be cytostatic for virus infected cells and tumour cells.

There has been limited success in physically separating populations of elicited or activated macrophages and testing for different functions (Lee & Berry, 1977; Stuart, 1977). In order to identify the phenotype of cytostatic peritoneal cells, we have used a rosetting

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procedure which allows cells to be separated on the basis of surface antigens such as Ig, Thy-1, Ia and receptors for antigen–antibody complexes (FcR) and for activated complement components (C3R). The results of these studies are presented in this paper.

MATERIALS AND METHODS

Mouse peritoneal exudate cells (PEC)

Peritoneal exudates were induced by intraperitoneal injection of 2 ml of 2% boiled and autoclaved potato starch (Hopkins & Williams, London) in Dulbecco's phosphate-buffered saline (Ca^{2+}, Mg^{2+}-free) into 6–9 week old male BALB/c mice obtained from the Imperial Cancer Research Fund animal breeding unit, London. PEC ($4–8 \times 10^6$/mouse) were collected 4 days after injection by peritoneal lavage with cold PBS. The cells were centrifuged at 400 ***g***, 4° and resuspended in Dulbecco's modified Eagle's medium containing 100 units/ml penicillin and 100 μg/ml streptomycin (E4; Cat. No. 188G, GIBCO Europe, Glasgow, Scotland), 5% foetal calf serum (FCS) and 15 mM HEPES (Hopkins & Williams, Romford, Essex), pH 7·2 (E4-H-5% FCS). Any exudates containing red cells were discarded. A selection for macrophages was frequently made by allowing PEC to adhere to petri dishes coated with BHK cell microexudate as described by Ackerman & Douglas (1978). The proportion of macrophages was assessed by staining for non-specific esterase enzyme (NSE) (Yam, Li & Crosby, 1971).

Cell lines

The K31 cell line (Aaronson & Weaver, 1971) was cultured in E4 containing 10% FCS and YAC-1, obtained from Dr Rolf Kiessling, Stockholm, was cultured in RPMI 1640 medium (Cat. No. 10-601-24, Flow Labs, Ayrshire, Scotland) containing 10% FCS. Sheep and ox red blood cells were routinely obtained from GIBCO Europe, Glasgow, and were used within 2 weeks.

Antisera

A.TH anti-A.TL serum (As 749, anti-I^k serum) was obtained from Dr I. F. C. McKenzie, Melbourne. This antiserum reacts with the Ia.7 and Ia.15 specificities shared between I^k and I^d mice. It had a cytotoxic titre on BALB/c spleen cells (I^d) of 1:1000 and was used in the reported experiments at 1:100 after titration by rosetting (see below) on PEC. Two inappropriate sera were used as controls. These were normal BALB/c serum and an anti-I-J^k serum, As 766B [B10.HTT × BALB/c F_1 anti-B10.S (9R)] obtained from Dr I. F. C. McKenzie. Serum containing Thy-1.2 monoclonal antibody was obtained from Dr Phil Lake, University College, London. It had a cytotoxic titre of $1:10^6$ against thymocytes and was used at a dilution of 1:100. Preparation of sheep anti-mouse immunoglobulin serum (Sh anti-MIg) and the pepsin digested derivative, Sh $F(ab')_2$ anti-MIg, has been previously described (Parish & McKenzie, 1978). All of the above sera were centrifuged at 100,000 ***g*** for 50 min immediately prior to use in any experiment in order to remove Ig aggregates.

Both rat anti-SRBC (IgM) and rat anti-ox red cells (IgG) were prepared and titrated by rosetting against mouse spleen cells as previously described (Parish & Hayward, 1974; Parish, 1975; Parish & Chilcott, 1975).

Red cell sensitization (EA, EAC, E-anti-MIg)

To sensitize ox red cells in order to detect Fc receptors (EA), a 5% suspension of ox red cells was incubated with an equal volume of rat anti-ox red cell serum at a dilution of 1:4 at 37° for 30 min. To sensitize sheep red cells in order to detect complement receptors (EAC), 5% sheep red cells were sensitized with an equal volume of rat anti-SRBC serum at a dilution of 1:80 at 37° for 30 min followed by incubation with mouse serum as a source of complement at 1:10 at 37° for 30 min. A control preparation of sensitized sheep cells was incubated without mouse serum (EA.IgM). Sh anti-MIg was coupled to SRBC by means of $CrCl_3$ (E-anti-MIg). This was done by mixing 0·25 ml packed SRBC, 1·2 mg Sh anti-MIg, 0·2 ml of 0·1% $CrCl_3$ in 4·0 ml saline for 5 min at room temperature followed by two washes in PBS.

Detection of rosetting cells

Cells bearing C3 and Fc receptors, Ig, Ia and Thy-1 antigens were detected by rosetting procedures which have previously been described in detail (Parish & Hayward, 1974; Parish, 1975; Parish & Chilcott, 1975; Parish & McKenzie, 1978). In all experiments, controls were included in which PEC were incubated with either unsensitized sheep or ox cells in order to control for non-specific loss of PEC. In general less than 0·1% rosette-forming cells (RFC) were observed in these controls. All manipulations were carried out in E4-H-5% FCS. Briefly, the rosetting procedures were as follows:

C3R-bearing cells or EAC rosettes. 0·5 ml PEC (2×10^7/ml) and 0·5 ml of a 10% suspension of E_{SRBC} EA.IgM or EAC were incubated at 37° for 10 min followed by centrifugation at 500 *g* for 3 min. Sodium azide (final conc. 0·1%) was added to the supernatant and the rosette mixture was gently resuspended before Ficoll–Hypaque gradient separation of rosetted from non-rosetted cells.

FcR-bearing cells or EA rosettes. 0·5 ml PEC (2×10^7/ml) and 0·5 ml of a 10% suspension of E_{ox} or EA were incubated at 37° for 10 min, followed by centrifugation at 500 *g* for 3 min. After leaving the pellet at room temperature for a further 30 min, the rosette mixture was treated as were the EAC rosettes.

Ig-bearing cells plus Thy-1 and Ia-bearing cells. 0·5 ml PEC (2×10^7/ml) plus 0·5 ml of either medium alone (Ig^+), monoclonal anti-Thy-1.2 antibody (1:100), A.TH anti-A.TL serum (1:100) or control sera (see Antisera section) were incubated on ice for 30 min followed by two washes with cold medium. These samples were resuspended in 0·5 ml cold medium and mixed with 0·5 ml of a 10% suspension of ice cold E-anti MIg cells. After a few minutes, this mixture was centrifuged at 500 *g* at 4° for 3 min and treated as previously described.

Separation of rosetting and non-rosetting cells. The Ficoll–Hypaque procedure for separating rosetted and non-rosetted cells and the recovery of rosetted cells by osmotic shock have been described (Parish, 1975; Parish, Kirov, Bowern & Blanden, 1974). Separated PEC were regularly more than 98% viable after these procedures.

Cytostatic assay

The cytostatic activity of PEC was assessed as previously described (Goldman & Hogg, 1977). Briefly, PEC were incubated with K31 target cells (5×10^3 cells per well) at effector:target cell ratios of 40:1 to 5:1 for approximately 18 h. The cells were washed once and pulsed with 0·5 μCi ^{125}IUdR (Radiochemical Centre, Amersham) per well for 4–5 h. After further washing, the plates were dried and the individual wells were cut out and counted in a Wallac gamma counter.

RESULTS

Proportions of FcR, C3R, Ig, Ia and Thy-1 bearing PEC

Peritoneal cells from mice which had been stimulated intraperitoneally four days previously with starch were fractionated using rosetting techniques into subpopulations bearing the surface markers, Fc or C3 receptors (R), surface Ig, *I*-region associated antigens or Thy-1 antigens. The percentage of PEC forming each type of rosette is given in Table 1. Approximately

Table 1. Percentage of starch-activated PEC bearing the surface membrane antigens, FcR, C3R, Ig, Ia and Thy-1 detected by rosetting techniques

Type of rosette	Percentage of rosetting cells ±SE*	No. of experiments	Average recovery (%) ±SE‡
E_{OX}	<0·1	6	68·8±9·7
EA (FcR+)	75·3±3·8	6	79·6±21·5
E_{SRBC}	<0·1	3	80·8±18·3
EA.IgM	<0·1	3	76·0±10·0
EAC (C3R+)	26·0±2·8	3	82·7±12·0
E_{SRBC}	<0·1	8	86·5±10·5
E-aMIg (Ig+)†	18·1±7·1	8	78·2±12·1
E-aMIg+anti-Ia (Ig− and Ia+)	47·2±9·7 (29·1)	8	70·0±13·8
E-aMIg+anti-Thyl (Ig− and Thyl+)	23·7±2·3 (5·6)	2	65·0±15·2

* PEC >4 SRBC were scored as rosettes.

† Sh $F(ab')_2$ aMIg was coupled to SRBC in two experiments.

‡ Recovery of PEC (both rosetting and non-rosetting cells) following separation on Ficoll–Hypaque.

75·3% of PEC possessed FcR and 26% C3R. An average of 18·1% PEC rosetted with SRBC coupled with Sh anti-MIg or Sh F(ab')$_2$ anti-MIg (E-aMIg). When PEC were first incubated with A.TH anti-A.TL (anti-I^k) serum or with a monoclonal anti-Thy-1.2 serum, before mixing with E-aMIg cells the percentage of rosettes increased to 47·2% and 23·7%, respectively. An initial incubation with inappropriate mouse sera did not raise the proportion of rosettes above that obtained with E-aMIg incubation alone (data not shown). Thus, there are 18·1% Ig^+, 29·1% Ia^+ Ig^-, 5·6% Thy-1^+, Ig^- as well as 75% FcR and 26% C3R bearing cells in the peritoneal exudates examined. The recovery of PEC following fractionation of rosetting and non-rosetting cells on Ficoll–Hypaque was usually 70–85% (Table 1). There was no indication that there was an increased loss of cells during any particular rosetting procedure.

Percentage of Ia^+ peritoneal macrophages

Generalized cytoplasmic staining for the non-specific esterase enzyme is considered to be a marker for cells of the monocyte/macrophage lineage. Approximately 77·6% of unfractionated cells were NSE positive (Table 2). The unstained cells were smaller and lymphoid in appearance, as were the majority of cells forming rosettes with E-aMIg (Ig^+) (0·5% < NSE^+). Pre-incubation with anti-Ia serum caused rosetting of a population of cells which were 15·8% NSE positive. These NSE+ cells were all large with the morphological appearance of macrophages and must be an Ia^+, Ig^- population as the Ig^+ cells were NSE^- (Table 2). Since the Ia^+, Ig^+ population represented in this experiment is only 45·5% of total PEC (Table 2), then the proportion of total PEC which is both Ia^+ and NSE^+ is 7·2% (15·8 × 45·5/100). Moreover, since the Ia^+, Ig^- population constitutes 70% (30·6/45·5 × 100) of the Ia^+ Ig^+ population, then approximately 54% (70–15·8) of the Ia^+ Ig^+ cells, or 25% of total PEC (54·2 × 45·5/100) are Ia^+ cells which are not mature macrophages (NSE^-), T cells (Thy-1^-) or B cells (Ig^-).

Table 2. The percentage of non-specific esterase (NSE) positive cells in unfractionated and rosetted populations

PEC	NSE positive cells (%)	Rosetting cells (%)
Unfractionated*	77·6	—
Fractionation by rosetting†		
Ig^- cells	98·7	85·1
Ig^+ cells	< 0·5	14·9
$Ig^+ + Ia^+$ cells	15·8	45·5
($Ig^- - Ia^+$)		(30·6)

* PEC prepared by adherence to microexudate-coated plates.

† Ig^-, Ig^+ and $Ig^+ + Ia^+$ cell populations purified by rosetting and Ficoll–Hypaque fractionation.

Surface antigen phenotype of cytostatic PEC

The PEC after being fractionated by rosetting into subpopulations bearing or lacking specific cell surface markers were tested for cytostatic activity against a tumour cell line, K31, which has previously been shown to be sensitive to macrophage-mediated cytostasis (Goldman & Hogg, 1977). FcR^+ PEC display comparable cytostatic activity to control PEC exposed to unsensitized SRBC and to unfractionated PEC (Fig. 1). The FcR^- PEC were virtually inactive. Similarly $C3R^+$ PEC were as cytostatic as control PEC (Fig. 2) and $C3R^-$ PEC were very much less cytostatic. Therefore, the cytostatic cell bears Fc and C3 receptors. In Fig. 3, the cytostatic activities of PEC fractionated according to the presence of Ig, and Ia or Thy-1 are presented and compared to a control preparation of PEC exposed to uncoupled SRBC. In all cases, the rosetted cells lacked cytostatic activity and the non-rosetted cells, lacking Ig, Ia and Thy-1 antigens, were as cytostatic as control PEC.

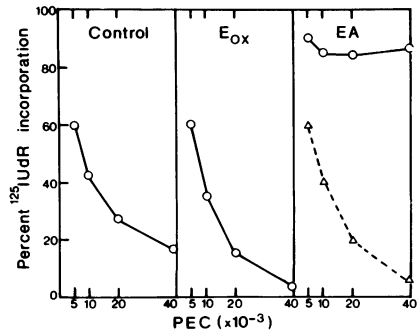

Figure 1. Cytostatic activity of FcR^+ and FcR^- populations of PEC. Non-rosetting cytostatic cells (○) are shown in Control, E_{ox} and EA fractionations, rosetting cells (△) are shown only in the EA fractionation. In general, less than 0·1% rosette-forming cells were observed in the control. ^{125}IUdR incorporation in control target cells (±SD) was 29,615 ± 1226 c.p.m. FcR^+ rosetting cells were 71·2% of total PEC.

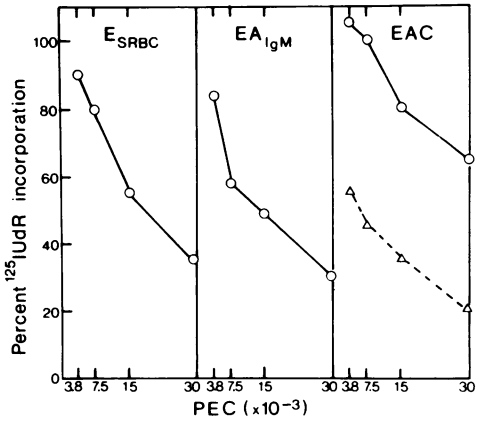

Figure 2. Cytostatic activity of C3R$^+$ and C3R$^-$ populations of PEC. Non-rosetting cytostatic cells (o) were found in E_{SRBC}. EA_{IgM} and EAC fractionations, and rosetting cells (Δ) in the EAC fractionation. ^{125}IUdR incorporation in control target cells (±SD) was 38,750±2486 c.p.m. C3R$^+$ rosetting cells (EAC) were 28·6% of total PEC.

DISCUSSION

In this study, we have examined the surface membrane markers of the cytostatic PEC elicited by starch. Using rosetting techniques, PEC were demonstrated to be 75% FcR$^+$, 26% C3R, 18% Ig$^+$, 29% Ia$^+$, Ig$^-$ and 5% Thy-1$^+$, Ig$^-$. By separating rosetting from non-rosetting cells using Ficoll–Hypaque gradients, cytostatic activity could be located in fractions bearing or lacking a particular surface marker. The cytostatic cell possessed a receptor for Fc (Fig. 1). Furthermore, if murine sera (e.g. anti-Ia) were not subjected to high speed centrifugation before use, complexes formed by freeze-thawing bound to FcR at dilutions up to 1:40,000 (data not presented) suggesting that these PEC possess unusually avid FcR. Cytostatic activity was found amongst the PEC which possessed a C3R (Fig. 2). Further analysis showed the cytostatic cell to lack Ig and Thy-1 making it unlikely to be related to T cells or to the type of cytostatic B cell described by Nathan and co-workers (Nathan, Hill & Terry, 1976; Nathan, Asofksy & Terry, 1977). Thus the active cell possesses both FcR and C3R and contains NSE (Table 2), properties which are characteristic of mature cells of the monocyte/macrophage series. Analysis of Mph obtained by tissue culture of bone marrow cells also shows that the ability to lyse tumour cells directly without antibody is a characteristic of morphologically more mature Mph (Domzig & Lohmann-Matthes, 1979).

When considered as a proportion of total NSE positive cells, macrophages were 7·2% Ia positive (Table 2) but the cytostatic cell was not in this population (Fig. 3). Therefore, the cytostatic marcrophage which lacks Ia differs from the type of macrophage involved in antigen presentation to T cells which is Ia positive (Shevach & Rosenthal, 1973; Schwartz & Paul, 1976; Erb *et al.*, 1976). It also should be noted that antigen-presenting macrophages need to express both *I-A* and

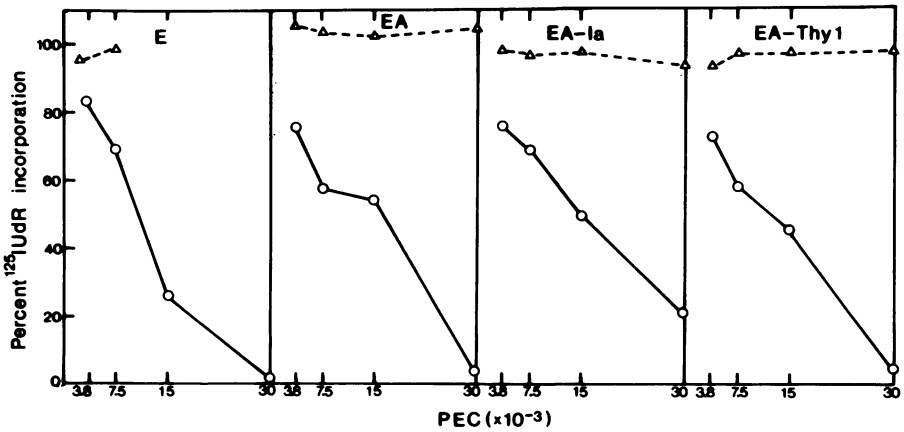

Figure 3. Cytostatic activity of Ig, Ia and Thy-1 fractionated populations of PEC. The cytostatic activities of non-rosetting (o) and rosetting cells (Δ) are shown for E, EA, EA-Ia and EA-Thy-1 fractionations. ^{125}IUdR incorporation in control target cells (±SD) was 56,088±2561 c.p.m. Rosetting cells were 14·9% Ig$^+$, 45·5% Ig$^+$Ia$^+$ (30·6%Ia$^+$) and 22·3% Ig$^+$ Thy-1$^+$ (7·4% Thy-1$^+$).

I-E/C subregion controlled Ia antigens to activate T cells successfully (Cowing *et al.*, 1978; Ahmann *et al.*, 1978; Hodes *et al.*, 1978). The anti-Ia serum used in this study detected both *I-A* (Ia.15 specificity) and *I-E* (Ia.7 specificity) subregion specificities in BALB/c mice. This provides evidence for the first time that cells of the monocyte/macrophage series with differing antigenic profiles perform different functions. It has been shown that the less mature human myeloblasts and promyelocytes bear Ia-like antigens which are not present on the mature granulocytes (Winchester, Ross, Jarowski, Wang, Halper & Broxmeyer, 1977). If Mph express Ia at a similar stage then the Ia^+ antigen presenting Mph represents a less mature cell than the Ia^- cytostatic Mph.

A second population of Ia^+ cells which were not mature macrophages (NSE^-), T cells ($Thy\text{-}1^-$) or B cells (Ig^-) constituted 25% of total PEC (Table 2). At present, further characteristics of these cells are unknown. In this context, there is some evidence in man of Ia^+ null cells (Winchester, Meyers, Broxmeyer, Wang, Moore & Kunkel, 1978; Horwitz, Niaudet, Greaves, Dorling & Deteix, 1978), and a similar population of Ia^+ cells can be detected in mouse bone marrow (McKenzie & Parish, in preparation).

There remains, however, the more general question of whether Mph distinguishable by surface markers represent independent subsets of Mph deriving from an undifferentiated cell type or represent different activation or maturational stages of the same cell population along a predetermined pathway. More progress towards answering these questions will come when antigens which are Mph-specific are characterized and can be used to analyse cloned populations of macrophages.

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