Monoclonal antibody EBM/11: high cellular specificity for human macrophages

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SUMMARY  A monoclonal antibody, EBM/11, was raised against isolated human lung macrophages. Immunohistochemically this antibody reacted with freshly isolated lung macrophages and blood monocytes, mononuclear cells (presumptive macrophages) in sections of lung, skin, stomach, small and large bowel, pancreas, spleen, tonsil, placenta, liver, gall bladder, heart, thyroid, pituitary, brain, and peritubular and mesangial cells in kidney. Microglial cells and osteoclasts also labelled with EBM/11. The antibody reacted with cytoplasmic structures rather than with cell membranes. The epitope recognised by EBM/11 was present on four polypeptides (of 120, 70, 64 and 22 kilodaltons). It did not react with any other cell type in the tissues screened except the epithelium of renal proximal tubules.

This antibody may be useful in identifying and elucidating the function of macrophages in pathological processes.

There is a need for reliable and sensitive reagents for the identification of human macrophages, particularly in studies of lymphoproliferative disease, the immune response, inflammation and neoplasia. To this end α-1-antitrypsin (α1-AT), 1 lysozyme, 2 peanut agglutinin (anti-T lectin), 3 and non-specific esterase 4 have been used as specific markers for macrophages. All of these, however, are found in cells which are manifestly not macrophages. For example, α1-AT is present not only in macrophages but also in polymorphs 5 and in hepatocytes. 6 Several monoclonal antibodies to macrophages/monocytes have been produced to overcome the shortcomings of histochemical methods for macrophage/monocyte identification. 78 Most of these have been used in studies of isolated monocytes but it is clear that many of them label cells other than macrophages/monocytes. 910

The aim of this study was to produce monoclonal antibodies to macrophages and to determine their specificity for this cell type. The long term objective was to use these reagents with high specificity for macrophages to elucidate macrophage functions in physiological and pathological states.

Material and methods

Preparation of macrophages
Specimens of fresh human lung resected for carcinoma were obtained from the Churchill Hospital, Oxford, and the East Birmingham Hospital. Lungs were transported to this laboratory in supplemented Leibovitz medium (L15). The supplements consisted of 10 mmol/l L-glutamine, 100 μg/ml each of benzylpenicillin and streptomycin, and gentamycin 80 μg ml−1 (Warrick, UK). Unless otherwise specified, all cell culture reagents were obtained from Gibco Biocult (UK).

About 50 g of macroscopically normal lung tissue distant from the tumour was minced thoroughly with scissors with as little L-15 medium as was practicable. The disaggregated lung tissue was mixed with an equal volume of L-15 medium, degassed carefully in a 1000 ml beaker in a vacuum desiccator at 760 torr after a method similar to that of Mason et al. 7 The component cells were isolated by squeezing the mince through sterile surgical gauze, then by filtering the eluted cells in L-15 medium through a steel tea strainer of large pore size. This cell isolation procedure was repeated once more on the disaggregated tissue, and the cells from both isolates were pooled. The mince was subsequently extracted similarly with L-15 medium additionally supplemented with 6 mmol/l edetic acid (Sigma, UK), 5% fetal calf serum, 10 mmol/l lignocaine hydrochloride (Astra, UK), and enough 0·1 mmol/l sodium bicarbonate (BDH, UK) to bring the pH to 7·0. Cells extracted with both media were immediately centrifuged at 120 g for 10 min in 50 ml Falcon tubes. Both cell pellets were pooled, resuspended in fresh L-15 medium containing antibiotics only placed on a Ficoll-Hypaque gradient (1·077 g), and centrifuged at 400 g for 20 minutes. The cells which
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concentrated at the interface were removed, washed, and resuspended at 10^6 cells/ml in L-15 medium, and placed in 80 cm^2 culture flasks (Nunc, Denmark) in 80 ml of RPM1 1640 medium supplemented with 5% fetal calf serum, benzylpenicillin (100 μg/ml), streptomycin (100 μg/ml), and gentamycin (80 μg/ml). After incubation in a 5% carbon dioxide and 95% oxygen atmosphere for one hour at 37°C the adherent monolayer was agitated and washed gently with two changes of the same medium, thus affording virtually pure macrophage monolayers. Macrophages were detached from the plastic with a sterile rubber policeman. With very fresh lung specimens, a total yield of 6 × 10^6 adherent macrophages/50 g of lung tissue was typical, though with lung samples of up to four or five hours old (transported in L-15 medium) the total yields were typically reduced to 3 × 10^6 adherent macrophages/50 g lung. These macrophages were judged viable according to their ability to exclude trypan blue.

MONOCLONAL ANTIBODY PRODUCTION AND SCREENING

About 10^7 purified macrophages in 0.2 ml Hanks’s balanced salt solution (BSS) were mixed with an equal volume of complete Freund’s adjuvant and injected subcutaneously into Balb C mice. The animals received three intraperitoneal booster doses of 10^7 cells in 0.2 ml of BSS, without adjuvant, at two week intervals. Three days after the final injection separated mouse spleen cells (about 10^8 cells) were filtered through a sterile stainless steel strainer and fused with 10^7 mouse myeloma cells (NSI) using 50% polyethylene glycol 4000 (BDH, UK) in RPM1 1640 medium; the cell suspension was agitated for 90 seconds and incubated for a further five minutes at 22°C. After immediate washing with RPM1 1640 containing hypoxanthine, methotrexate, and thymidine (HAT) the cells were resuspended in 50 ml of RPM1 1640 containing HAT and 10% fetal calf serum, and plated into Costar wells. This method afforded between 300 and 400 hybrid colonies. Two hundred of these colonies were separated, subcloned, grown and frozen in liquid nitrogen.

As a primary screen for macrophage reactive antibodies culture supernatants were tested by an indirect immunoperoxidase procedure on cryostat sections of normal lung, tonsil, lymph node, spleen, liver, pancreas, stomach, small and large bowel, gall bladder, kidney, heart, brain, pituitary, thyroid, placenta, and skin. Cytocentrifuged buffy coat cells from human peripheral blood and isolated lung macrophages fixed in acetone were also included in this secondary screen. All tissues and cells used in this secondary screen were obtained fresh at biopsy, or from necropsy organs within 24 hours of death.

Immunohistochemical controls consisted of tissues or cells treated with culture medium alone, or irrelevant monoclonal antibodies to Mallory’s bodies (JMB1, JMB2), followed by rabbit antimouse Ig conjugated with peroxidase (Dako, UK), or, in a few instances, rabbit antimouse followed by alkaline phosphatase-mouse antialkaline phosphatase complex. The conditions for immunohistochemical analysis are as described, except that after the application of primary antibody (or control antibody) sections or cells for immunoperoxidase staining were treated for 30 minutes with methanol containing 1% H2O (v/v) at 22°C and washed in PBS before incubation with second antibody. In some tissues the reaction of EBM/11 was detected using the alkaline phosphatase-antialkaline phosphatase method using a standard procedure.

The immunoglobulin class of interesting monoclonal antibodies was determined by the Ouchterlony method using subclass specific antisera (Dako, UK).

WESTERN BLOTS

Lung macrophages were isolated and maintained for four to 24 hours in L-15 medium supplemented with antibiotics (see above) and 10% heat inactivated fetal calf serum. Cultures were washed twice in Hanks’s BSS, harvested with a rubber policeman, and the macrophages recovered by centrifugation at 1500 g for five minutes. The cell pellet was dispersed in a minimum volume of a buffer solution containing Triton-X100 (0.1%), 5 mmol/l edetic acid, 0.01 mmol/l Tris-hydrochloric acid, 2 mmol/l phenylmethylsulphonyl fluoride, 2.5 mmol/l iodoacetamide of pH 7.4. The cell suspension was sonicated for 30 seconds and centrifuged at 1500 g for 15 minutes. Ten volumes of absolute ethanol were added to the supernatant, incubated at 22°C for five minutes, and the precipitated protein recovered by centrifugation (12 000 g for five minutes). The pellet was dissolved at 80°C for five minutes in 5% sodium dodecyl sulphate (SDS) containing 2% mercaptoethanol. Proteins were electrophoresed on 7.5% polyacrylamide gels in an electrophoresis buffer containing 80 mmol/l Tris, 50 mmol/l boric acid, and 0.1% SDS at pH 8.5; molecular weight standards were run on each gel. Proteins were transferred (in an electric field for 16 hours) to nitrocellulose filters (0.45 μm pore size) using a buffer
Macrophages in alveolar air spaces and some alveolar lining cells are EBM/11 positive. EBM/11 reaction is cytoplasmic and granular. EBM/11 reactivity was visualised by indirect immunoperoxidase histochemistry (unless otherwise stated). Sections are counterstained with haematoxylin.

Fig 1

Hepatic Kupffer cells and mononuclear cells in portal tracts are EBM/11 positive. Reaction product is granular. Brown granules in hepatocytes are lipofuchsin.

Fig 2

Lymph nodes: interdigitating reticulum cells, sinus macrophages, and germinal centre macrophages are EBM/11 positive. (APAAP procedure.)

Fig 3

Macrophages in gastric lamina propria are EBM/11 positive.

Fig 4

Results

Twenty monoclonal antibodies in the primary screen reacted with pulmonary and tonsillar macrophages. Of these, 18 reacted with other cell types in the multiorgan screen; in general, these antibodies reacted not only with macrophages but also with stratified squamous epithelia, other epithelia or polymorphs and lymphocytes, or a combination. Of the remaining two antibodies, one, designated EBM/11, reacted with almost all macrophages and monocytes, and the other only with lung macrophages. The characteristics of the other antibody will be described elsewhere.

EBM/11 reacted with isolated alveolar macrophages and with the same cells in intact lung (fig 1). Kupffer cells in liver (fig 2), and littoral cells in splenic
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Fig 5 Epidermal Langerhans' cells and mononuclear cells in upper dermis are EBM/11 positive (APAAP procedure.)

Fig 6 Renal peritubular mononuclear cells are EBM/11 positive.

Fig 7 All monocytes in this buffy coat preparation from peripheral blood are EBM/11 positive. All other white cells are EBM/11 negative.

Fig 8 Invasive ductal cancer of breast. Malignant cells are EBM/11 negative. Many mononuclear stromal cells are EBM/11 positive.

Table Distribution of EBM/11 positive cells in normal tissues

<table>
<thead>
<tr>
<th>Organ</th>
<th>Cell types</th>
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<tbody>
<tr>
<td>Lung</td>
<td>Alveolar macrophages</td>
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<tr>
<td>Liver</td>
<td>Kupffer cells, portal tract mononuclear cells</td>
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<tr>
<td>Gall bladder</td>
<td>Macrophages</td>
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<tr>
<td>Skin</td>
<td>Dermal macrophages; Langerhans' cells</td>
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<tr>
<td>Brain</td>
<td>Microglial cells; perivascular mononuclear cells</td>
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<tr>
<td>Kidney</td>
<td>Mononuclear cells around renal tubules; mesangial cells in glomeruli</td>
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<tr>
<td>Spleen</td>
<td>Macrophages and dendritic cells in red and white pulp</td>
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<tr>
<td>Lymph nodes</td>
<td>Interdigitating reticulum cells; sinusoidal and germinal centre macrophages</td>
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<tr>
<td>Tonsil</td>
<td>Macrophages (B and T cell zones)</td>
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<tr>
<td>Stomach</td>
<td>Macrophages in vili and mononuclear cells in circular and longitudinal muscle coats</td>
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<tr>
<td>Ileum</td>
<td>Mononuclear cells between muscle bands</td>
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<tr>
<td>Colon</td>
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<td>Heart</td>
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<td>Pancreas</td>
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<tr>
<td>Stromal mononuclear cells</td>
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<tr>
<td>Thyroid</td>
<td>Mononuclear cells around follicles</td>
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<tr>
<td>Pituitary</td>
<td>Nonendocrine mononuclear cells</td>
</tr>
<tr>
<td>Buffy coat</td>
<td>Monocytes only</td>
</tr>
<tr>
<td>Bone</td>
<td>Osteoclasts, mononuclear cells, megakaryocytes</td>
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mononuclear/phagocyte system such as Kupffer cells, sinusoidal cells of spleen, and alveolar macrophages. It also labels cells with a macrophage morphology around renal tubules and thyroid follicles, non-endocrine cells of the pituitary, and Langerhans’ cells of the epidermis; all of these cells have now been included in the mononuclear/phagocyte system as defined by their reactivity with F4/80 in mouse tissue. In man, there is phenotypic evidence that Langerhans’ cells belong to the mononuclear/phagocyte system. Franklin et al estimated that 90% of Langerhans’ cells stained with EBM/11 if HLA-DR positivity is taken as the standard. Interestingly microglial cells were positive with EBM/11. Del Rio Hortego assumed that microglial were part of the mononuclear/phagocyte system. Mouse microglia also reacts with F4/80. Some glomerular mesangial cells reacted with EBM/11; there is corroborative evidence that some mesangial cells are part of the mononuclear/phagocyte system. EBM/11 has been shown to react with osteoclasts, megakaryocytes, and marrow mononuclear cells. These three cell types therefore have a phenotypic similarity to macrophages. Additionally, all of them react with other antibodies which have broad specificity for macrophages. Athanassou discusses the association between osteoclasts, megakaryocytes, and mononuclear phagocyte system cells.

The molecular and cellular nature of the antigen with which EBM/11 reacts is now the subject of investigation. The data from Western blotting indicate that it reacts with four major polypeptides from isolated lung macrophages of relative molecular weights 120, 70, 64 and 22 kd. These polypeptides differ in mass from those identified by other antimacrophage monoclonals. The functions of these polypeptides are not yet known. EBM/11 also differs from most other monoclonal antibodies to macrophages in staining intracytoplasmic structures which have a granular appearance (fig 7) on light microscopy; many other antimacrophage monoclonals react with cell membrane components. Preliminary ultrastructural evidence indicates that the antibody may react with rough or smooth endoplasmic reticulum; it does not react with mitochondria, Golgi apparatus, or lysosomes (PMA Kelly, I Ferguson, and J O’D McGee, unpublished observations). The similarities and differences of EBM/11 reactivity compared with other macrophage monoclonals is discussed at length by Franklin et al. At the Third International Workshop on Human Leucocyte Differentiation Antigens, EBM/11 was assigned to group 12, the mixed antimacrophage group. Within this group it is included in subgroup 4, which includes those antibodies with broad specificity. Unlike many of the antibodies in this group, it shows strong reactivity with
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Peripheral blood monocytes.

The screening procedure used here for the generation of EBM/11 is instructive. Although 20 monoclonal antibodies were produced which reacted with the primary immunogen (lung macrophages), only one of these showed high cellular specificity for cells of the mononuclear/phagocyte system. This emphasises the importance of screening monoclonal antibodies against large numbers of intact tissues before claims are made for cellular specificity. We have shown in this paper that EBM/11 labels known or presumptive cells of the human mononuclear/phagocyte system in normal tissues with a high degree of specificity.

It has also been shown to label multinucleate cells in giant cell myocarditis and in giant cell tumour of bone. The tumour cells in a single case of malignant histiocytosis (unpublished observations), and also presumptive macrophages in human breast cancer (fig 8). There has been controversy about the nature of these cells in these various diseases. We believe that the demonstration of the shared relatively specific EBM/11 positive immunophenotype between these cells and normal mononuclear/phagocyte system cells has contributed to the understanding of these diseases and that this antibody will assist in the study of other disease processes in man.

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References

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