A new monoclonal antibody (3A5) that recognises a fixative resistant epitope on tissue macrophages and monocytes

E H Jaspars, E Bloemena, P Bonnet, R J Scheper, E Kaiserling, C J L M Meijer

Abstract

Aims—To develop a monoclonal antibody specific for human macrophages in routinely processed material.

Methods—The monoclonal antibody was derived from a mouse popliteal lymph node after subcutaneous immunisation in the footpad with fragments of human spleen depleted of lymphocytes and erythrocytes.

Results—3A5 is a monoclonal antibody reactive with macrophages, monocytes, and histiocytes in routinely processed (formalin fixed, paraffin wax embedded) human tissue specimens. Unlike the well known panmacrophage marker KP1 (CD68), neither dendritic cells (interdigitating cells, Langerhans' cells, and microglia) nor myeloid, lymphoid, or epithelial cells stained with 3A5.

Conclusion—As the staining pattern of 3A5 is restricted, compared with other macrophage markers and the recognised epitope survives common fixation and embedding procedures, 3A5 is a valuable marker for histiocytes and macrophages in routine diagnostic applications.

(*J Clin Pathol* 1994;47:248-252)

Macrophages are important to the immune system: they are the main effector cells in the innate immune response and are also indispensable in adaptive immunity because they process antigen, secrete soluble factors, and guide lymphocytes in their essential recirculation.^{1,2} Unlike the numerous antigens on T and B lymphocytes which have been identified over the past few years, specific markers for human macrophages are rare and not yet well characterised. An important reason for this is that the mononuclear phagocyte system (MPS) is morphologically and functionally diverse, and the differentiation state does not always correlate with the phenotypic appearance nor with the function of the cell.

Several antibodies have been raised against monocytes/macrophages, but most of them do not recognise all the cells or are not lineage specific. Recently, CD68 was introduced by the Fourth International Workshop on Leucocyte Differentiation Antigens as a 110 kilodalton antigen, reactive with MPS cells.³ Ever since, several anti-CD68 monoclonal antibodies have been described which differ subtly in their immunohistochemical staining patterns, possibly because of variations in gly-

cosylation of the CD68 protein in different cell types.^{4,5} The functional aspects of CD68 are still unclarified, mainly due to its localisation in lysosomal granules,⁶ which hinders functional studies with antibodies.

Besides the need for lineage specific antibodies in fundamental research, pan-macrophage markers are also of great value in diagnostic pathology. Because of the great morphological heterogeneity in macrophage cells, a clear distinction from other cells can be extremely difficult, especially when the macrophages have undergone malignant transformation. Among the expanding panel of anti-CD68 monoclonal antibodies KP1 is one of the most widely used in routine diagnostic pathology, merely because it stains cells on paraffin wax embedded tissues.⁷ However, besides macrophages this monoclonal antibody also reacts with myeloid cells,⁸ "plasmacytoid" T lymphocytes,⁹ and epithelial cells in certain disorders.^{10,11}

Methods

PRODUCTION OF MONOCLONAL ANTIBODIES

A spleen specimen, surgically removed because of a vena lienalis thrombosis, was depleted of lymphocytes and erythrocytes by cutting and squeezing it through a nylon gauze. The remaining substance was fragmented by a Polytron blender, suspended in phosphate buffered saline (PBS), emulsified in Freund's Complete Adjuvant (1 in 1), and injected subcutaneously (three times) in the footpads of Balb/C mice (10 µl/footpad). Four days after the last injection, lymphocytes from the popliteal lymph nodes were fused with Sp20-Ag14 mouse myeloma cells, following standard techniques, as described before.¹³ The supernatant fluids were initially screened on cryostat sections of human spleen and tonsil for immunohistochemical reactivity with macrophages. Further testing was performed on paraffin wax embedded material. The selected hybrid (3A5) was subcloned three times.

For double staining (see below), the following monoclonal antibodies were used: the anti-CD68 KP1, commercially obtained from Dako reagents (Copenhagen, Denmark), and PG-M1, kindly provided by Dr B Falini (Perugia, Italy).

CELLS AND CELL LINES

Routine bone marrow smears taken for diagnostic purposes were obtained from the Pathology Department of Westeinde

Department of Pathology, Free University Hospital, Amsterdam, The Netherlands

E H Jaspars
E Bloemena
P Bonnet
R J Scheper
C J L M Meijer

Department of Special Histopathology and Cytopathology, Institute of Pathology, University of Tübingen, Germany
E Kaiserling

Correspondence to:
E H Jaspars, Department of Pathology, Free University Hospital, De Boelelaan 1117, 1007 MB Amsterdam, The Netherlands.

Accepted for publication 14 October 1993

Hospital, The Hague, The Netherlands. The slides were air-dried and stored at -20°C. Before use they were fixed in acetone for 10 minutes. Peripheral blood mononuclear cells were prepared from anticoagulated blood by Ficoll-Hypaque density centrifugation. Monocytes were purified by counterflow centrifugation elutriation as described before.¹⁴

THP-1 (a monocytic leukaemia cell line), U-937 (a B cell lymphoma cell line with monocytic characteristics), K-562 (an erythro-leukaemia cell line with pluripotential differentiation abilities), and HL60 (a myeloid cell line) were cultured in RPMI 1640 containing 10% fetal calf serum, supplemented with 4 mM glutamin, 10 U/ml penicillin, and 0.1 mg/ml streptomycin.

Peripheral blood monocytes and cell lines were stimulated for 0.5, 1, or 2 hours in RPMI medium containing 100–500 U/ml interferon γ , 10–40 μ g/ml lipopolysaccharide (LPS), or 5–20 nM phorbol myristate acetate (PMA). Immunocytochemical staining of the cells was performed on cytopsin preparations, which were air-dried, fixed in acetone, and stored at -20°C.

FIXATION PROCEDURES

Most of the tissues were obtained from surgical procedures in our own clinic (Surgical Department, Free University Hospital, Amsterdam, The Netherlands). Some of the specific histiocytic lesions were kindly provided by Professor H Kerl (Gratz, Switzerland) and Dr M Santucci (Florence, Italy). Part of the tissue was snap frozen and stored in liquid nitrogen. Frozen sections (4 μ m) were cut, mounted on poly-L-lysine coated glass slides, dried and fixed in acetone for 10 minutes before performing immunohistochemistry. Other part of the tissues were fixed in Sensofix (Sensomed, Beuningen, The Netherlands), sublimate buffered formalin or formalin, and embedded in paraffin wax. Sections (4 μ m) were cut and stored at 4°C. Before immunostaining, the slides were dewaxed in xylol and rehydrated in a graded series of ethanol.

IMMUNOPEROXIDASE STAINING

For immunohistochemistry, a standard two-step immunoperoxidase staining procedure was performed as described before.¹⁵ To improve the staining results of 3A5, the formalin fixed tissues were preincubated with 10 mM citrate buffer (pH 6) for 5 minutes at 720 Watts in a microwave oven. Frozen sections and material fixed in Sensofix or sublimate buffered formalin needed no additional treatment. Isotype analysis was performed using isotype specific peroxidase conjugates (Serotec, Oxford, England).

For double staining the slides were first stained with the first monoclonal antibody by the two-step immunoperoxidase procedure using 3.3 diamino benzidine tetrahydrochloride as a substrate; the second monoclonal antibody was visualised by the alkaline phosphatase method, essentially as described.^{15 16}

Results

The hybridoma supernatant fluids were initially screened on frozen sections of human tonsil tissue and selected for specific reactivity with tissue macrophages. The selected antibody (3A5) also stained macrophages in paraffin wax embedded material and proved to be of the IgG2B subclass.

IMMUNOHISTOCHEMICAL REACTIVITY OF 3A5 ON TISSUE SECTIONS

Normal tissues

In lymph nodes, tonsils, and mucosa-associated tissue (MALT), 3A5 strongly stained the starry sky macrophages in germinal centres (fig 1), but also macrophages in T cell areas and in the sinuses. Follicular dendritic cells (FDC) and interdigitating cells (IDC) did not stain; neither did lymphocytes, myeloid cells, and other cell types. The white pulp of the spleen showed a comparable staining pattern with the other lymphoid tissues; red pulp macrophages were also positive. Furthermore, 3A5 reacted with most macrophages and histiocytes in all other tissues investigated (table 1). Remarkably, cells from the Langerhans-interdigitating cell series and microglia showed no reactivity.

Haemopoietic cells

In peripheral blood smears monocytes showed intracytoplasmatic staining with 3A5. Some reactivity was also observed within basophilic granulocytes, but no other cell types were positive (table 1). The immunocytochemical staining of peripheral blood monocytes was granular and rather weak. Expression of the 3A5 defined antigen could not be upregulated by exposing the cells to various concentrations of monocyte activating agents, like PMA, interferon γ , or LPS.

In bone marrow smears only macrophages and mast cells stained with 3A5. Megakaryocytes and myeloid cells did not, nor did other blood forming elements. Monocyte precursor cells could not be detected by the antibody.

Cell lines

Only the THP-1 monocytic line showed some

Table 1 Immunoreactivity of 3A5 on normal tissues

<i>Lymphoid tissue:</i>	
Tonsil/lymph node	Starry sky macrophages, interfollicular macrophages, sinus macrophages
Spleen	Macrophages in red and white pulp
Thymus	Medullary and cortical macrophages
Bone marrow	Macrophages, basophilic granulocytes and mast cells. No other haemopoietic cells
<i>Non-lymphoid tissue:</i>	
Lung	Alveolar macrophages
Kidney	Interstitial macrophages
Liver	Kupffer cells, periportal macrophages
Skin	Dermal macrophages (no Langerhans' cells)
Pancreas	Interstitial macrophages
Gut	Lamina propria macrophages
Testis	Interstitial macrophages
Uterus	Interstitial macrophages
Thyroid	Interstitial macrophages
Brain	Perivascular macrophages (no microglia)

Figure 1 Starry sky macrophages in a germinal centre of a reactive lymph node, stained by 3A5.

Figure 2 3A5 staining of reactive histiocytic cells in a lymph node (Hodgkin's disease); the Reed-Sternberg cell (centre) remains negative.

Figure 3 Reticulo-histiocytoma of the skin stained by 3A5.

reactivity with 3A5: the large cells stained strongly, but the smaller cells were only weakly positive or lacked the 3A5 defined antigen. The other cell lines (U937, HL60, and K562) did not react with the monoclonal antibody either unstimulated or after stimulation with several activating agents.

Diseased tissues

The distribution of the 3A5 defined antigen was investigated on a large collection of pathologically changed tissues (table 2).

Reactive lymphoid tissues—All sorts of reactive and inflamed lymph nodes were studied and most of the macrophage cells reacted. Besides the normal-looking macrophages epithelioid cells and multinucleated cells in granulomas also showed strong labelling. The dendritic cells in dermatopathic lymphadenopathy did not stain.

Malignant lymphoma—In Hodgkin's disease and non-Hodgkin's lymphomas only the macrophages reacted with 3A5 (including starry sky macrophages in Burkitt-like lymphomas). Neoplastic and reactive lymphoid cells did not stain. Particular attention was paid to the CD30 positive cells in anaplastic T cell lymphomas: they were negative as were Reed-Sternberg cells and Hodgkin cells in the different types of Hodgkin's disease (fig 2).

Leukaemia—Leukaemic cells from biopsy specimens of patients with chronic myeloid leukaemia or hairy cell leukaemia did not stain with 3A5.

Lesions of "histiocytic origin"—3A5 stained macrophages and histiocytic cells in a variety of inflammatory conditions and lesions of histiocytic origin (fig 3). Giant cells in benign and malignant giant cell tumours also reacted

Table 2 Immunoreactivity of 3A5 in pathologically changed tissue

<i>Reactive/inflamed lymph nodes</i>	
Follicular hyperplasia	Follicular, interfollicular, and sinus macrophages
Sinus histiocytosis	Follicular, interfollicular, and sinus macrophages
Rosai Dorfmann lymphadenopathy	Follicular, interfollicular, and sinus macrophages
Toxoplasmosis	Follicular, interfollicular, and sinus macrophages and epithelioid cells
Dermatopathic lymphadenopathy	Follicular, interfollicular, and sinus macrophages (no IDC)
<i>Malignant lymphomas</i>	
Non-Hodgkin's lymphomas	Macrophages, no lymphoma cells
Hodgkin's lymphomas	Macrophages, no lymphocytes, Reed-Sternberg, nor Hodgkin cells
<i>Benign lesions of histiocytic origin</i>	
Dermatofibroma	Reactive and neoplastic histiocytic cells
Granuloma annulare	Histiocytic cells
Xanthoma	Xanthoma cells
Xanthogranuloma	Xanthoma and giant cells
Sarcoidosis	Epithelioid and giant cells
Reticulohistiocytoma	Histiocytic cells and giant cells
<i>Malignant lesions of (putative) histiocytic origin</i>	
Histiocytosis X	Reactive histiocytes and macrophages, no tumour cells
Malignant fibrous histiocytoma	Reactive histiocytes and macrophages, no tumour cells
Malignant giant cell tumour	Macrophages and giant cells

Figure 4 A giant cell tumour of the synovium showing strong reactivity with 3A5 in the giant cells.

Figure 5 Immunohistochemical analysis of interfollicular cells in a lymph node reactive with 3A5 (brown) and KP1 (blue). In this experiment the slides were first incubated with 3A5. Note the variable double staining of some of the cells.

Figure 6 Immunohistochemical double staining of macrophages in a reactive lymph node, first incubated with 3A5 (brown), followed by PGM-1 (blue). G = germinal centre, I = interfollicular area.

with the monoclonal antibody (fig 4). However, the tumour cells in histiocytosis X and in the different variants of malignant fibrous histiocytoma lacked the antigen.

Mast cell disorders—Reactive and neoplastic mast cells in almost all cases (urticaria pigmentosa, cutaneous mastocytoma, systemic mastocytosis and malignant mastocytosis) stained, though the staining was much weaker than in macrophages and histiocytes.

Epithelial disorders—In sialadenitis, adenolymphoma of the salivary gland and hyperplastic polyps of the colon, 3A5 stained only macrophages and histiocytes. In contrast, KP1 showed reactivity with epithelial cells in these disorders, as has recently been described.¹⁰

IMMUNOHISTOCHEMICAL DOUBLE STAINING

To compare 3A5 with other macrophage markers—KP1 and PG-M1—double staining was done on tonsil tissue sections. When 3A5 was added as the first monoclonal antibody, followed by KP1, the different macrophage cells showed diverse staining patterns. In starry sky macrophages very little or no KP1 reactivity remained; interfollicular macrophages showed a variable, but clearly discernible, two-coloured intracytoplasmatic staining (fig 5). In this area some cells with dendritic morphology were only positive for KP1. When the slides were first incubated with KP1, no further reactivity with 3A5 was observed.

Double staining analysis with 3A5 and PG-M1, adding 3A5 first, showed that PG-M1 also stained some cells with dendritic morphology in the paracortex which were 3A5 negative, although less than KP1 (fig 6). However, no double staining cells were detected. When PG-M1 was added first in these experiments, no further 3A5 reactivity could be detected.

Discussion

3A5 is a highly specific monoclonal antibody for the macrophage lineage, as has been shown in an extensive immunohistochemical analysis of normal and diseased human tissues. Besides basophilic granulocytes and mast cells, it does not cross-react with other cell types. The detection of basophilic granulocytes and their tissue derivatives by 3A5, however, is not very surprising, as it has recently been shown that these cells are very closely related to the monocytic lineage.¹⁷ According to its tissue distribution, the 3A5 defined antigen is very likely to belong to the CD68 cluster. Compared with KP1, one of the most widely used anti-CD68 monoclonal antibodies,⁷ 3A5 shows a far more restricted staining pattern, excluding myeloid cells, dendritic cells (IDCs, Langerhans' cells, and microglia), lymphoid cells and epithelial cells. In immunohistochemical double staining experiments 3A5 and KP1 hinder each other from binding, suggesting that the monoclonal antibodies react with partly overlapping epitopes. Because 3A5 cannot block all KP1

reactivity in this sequential staining method, it seems that part of the KP1 antigen lacks a specific structure that is necessary for 3A5 recognition. Apparently, these KP1 positive, 3A5 negative molecules are variably present within the different macrophages of lymphoid tissue. As the 3A5 defined epitope is most strongly expressed in the more differentiated cells and its expression is not influenced by various activating agents, 3A5 may detect a differentiation rather than an activation molecule.

In immunohistochemical staining patterns 3A5 shows remarkable similarity with the macrophage-restricted anti-CD68 monoclonal antibody PG-M1.¹² Double staining experiments on tonsil and lymph node sections, however, indicated a small population of paracortical cells with dendritic morphology, not reactive with 3A5 and still PG-M1 positive. In contrast to 3A5, PG-M1 does not stain normal or reactive mast cells.¹⁸ Therefore, both monoclonal antibodies apparently do not recognise exactly the same epitope.

Like KP1 and PG-M1, the 3A5 defined epitope is resistant to routine fixation procedures, which makes it very useful for diagnostic pathologists. Moreover, 3A5 does not react with undifferentiated tumour cells other than those of histiocytic origin. The fact that histiocytosis X cells did not react can be explained by the fact that these tumours are derived from Langerhans' cells, which are also negative with the monoclonal antibody. No reactivity was observed in tumour cells of the different types of malignant fibrous histiocytoma. In spite of their name, however, the true origin of these tumours still remains highly controversial.^{19,20} Our findings support the idea that (at least part of) the malignant fibrous histiocytomas show no histiocytic differentiation.

These data led us to conclude that 3A5 is not only a highly specific marker for macrophages, useful in discriminating monocytes and macrophages from other cells of the mononuclear phagocyte system, such as myeloid cells and dendritic cells, but that it may also be of great value in routine diagnostics to distinguish histiocytes from tumour cells and for typing undifferentiated malignancies.

We thank Professor H Kerl and Dr M. Santucci for providing paraffin wax sections of histiocytic disorders, Dr B Falini for the gift of PG-M1, Miss PA den Otter for expert technical assistance and Dr P van der Valk for helpful comments.

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