Mac 387 antibody and detection of formalin resistant myelomonocytic L1 antigen

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SUMMARY The murine monoclonal antibody Mac 387 was raised against a purified protein fraction obtained from human monocytes. By immunoblotting experiments, Mac 387 was shown to react with a previously defined antigen called L1; this is a multichain myelomonocytic protein of about 36 Kd which shows sequence homology with the cystic fibrosis antigen. The L1 protein is present in the cytoplasm of virtually all resting peripheral neutrophils and monocytes; it is also variably expressed on the plasma membrane of these cells, possibly as a secretory product. Because the L1 antigen is resistant to denaturation by formalin, its tissue distribution can be studied in routinely processed biopsy material. In a wide variety of specimens Mac 387 was shown by immunohistochemical analysis, to produce a cytoplasmic staining pattern concordant with that of a well defined polyclonal antibody to the L1 antigen. Cytoplasmic reactivity was obtained with granulocytes and infiltrating macrophages but generally not with several categories of dendritic cells. In addition, squamous epithelium of mucous membranes was strongly positive, in contrast to normal epidermis.

The human L1 antigen is a multichain protein of about 36 Kd first isolated from granulocytes; it binds calcium ions avidly and shows a striking heterogeneity on isoelectric focusing.¹ Like lysozyme and myeloperoxidase, L1 occurs in both circulating neutrophils and monocytes.² It is of interest that the L1-light chain shows sequence homology with the cystic fibrosis antigen,^{3,4} and the two L1 chains seem to be identical with the recently described macrophage migration inhibitory factor (MIF)-related proteins MRP-8 and MRP-14.^{3,5}

The L1 antigen is remarkably well preserved in formalin fixed and paraffin wax embedded routine biopsy material.⁶ L1 shows a restricted distribution within the monocyte-derived cell lineage, being mainly confined to reactive histiocytes (infiltrating macrophages). It is usually lacking in HLA-DR positive interdigitating cells, Langerhans' cells, intestinal histiocytes, Kupffer cells of normal livers, and follicular dendritic cells.⁶ A protein sharing physicochemical and antigenic properties with L1 has been identified in psoriatic epidermal extracts.⁷ It is generally not expressed by normal epidermis but is abundant in several inflammatory skin diseases⁸ and adjacent to

various skin tumours.⁹ Moreover, mucosal squamous epithelium normally expresses L1.⁷

A rabbit antiserum S22, raised against human monocytes showed some similarities to anti-L1 when tested by immunohistochemical techniques.¹⁰ The S22-related antigens, however, were found to be expressed by Kupffer cells in normal livers and also by interdigitating cells. A similarly prepared antiserum S42 was recently used to capture detergent-solubilised monocyte proteins which were then used to raise a murine monoclonal antibody.¹¹ This study aimed to show that Mac 387 is specific for the well defined L1 antigen.

Material and methods

A variety of biopsy specimens were obtained from the routine pathology files.⁶ This material had been fixed in buffered formalin (pH 7.4) and embedded in paraffin wax. A similar selection of tissue was obtained as fresh material and prepared by direct ethanol fixation at 4°C followed by paraffin wax embedding according to the method of Sainte-Marie.¹²

PRIMARY ANTIBODY REAGENTS FOR IMMUNOHISTOCHEMISTRY

Production and characterisation of monoclonal Mac

387 have been detailed elsewhere.¹¹ Fluorescein and rhodamine conjugates were prepared from appropriately absorbed antisera with various specificities, including anti-L1.^{2,13,14} A rabbit antiserum to S-100 protein (A588, Dako, USA) was used to label Langerhans' and interdigitating cells.¹⁵ All antibody incubations took place at room temperature for 30 minutes or 20 hours; the reagents were appropriately diluted in isotonic phosphate buffered saline (PBS), pH 7.5, containing bovine serum albumin (BSA) at 125 g/l.

IMMUNOENZYME STAINING

Sections (4–6 μ m) of formalin fixed tissues were stained with or without trypsin (1.0 g/l) pretreatment for 10–15 minutes at 37°C. Antigen localisation was achieved by the alkaline phosphatase anti-alkaline phosphatase (APAAP) method,¹⁶ as detailed previously.⁶ Mac 387 was applied as ascitic fluid at dilutions of 1/1200 to 1/2400 for 20 hours on formalin fixed sections. The latter condition could likewise be used on ethanol fixed sections, but incubation for 30 minutes at 1/400 was preferable to avoid leaching out of cytoplasmic L1 antigen. Antiserum to S-100 protein was applied on formalin fixed sections at 1/1500 for 20 hours. After the final wash all sections were counterstained with haematoxylin.

PAIRED IMMUNOFLOUORESCENCE STAINING

A rhodamine labelled rabbit IgG (0.18 g/l) anti-L1 conjugate was applied on ethanol fixed tissue sections for 30 minutes and on formalin fixed sections for 20 hours. Mac 387 was used at 1/800, either mixed with the rhodamine conjugate or applied first in sequential incubations. Its reaction sites were finally shown by a two step method using biotinylated horse anti-mouse IgG and fluorescein labelled avidin.¹³ The red signal of the anti-L1 conjugate was enhanced by inclusion of rhodamine labelled swine anti-rabbit IgG in the last incubation step.¹³ The same detection system was used for paired staining with antiserum to S-100 protein (1/300) and Mac 387. The sections were evaluated in a Leitz Orthoplan microscope with a Ploem-type epilluminator.

IMMUNOBLOTTING EXPERIMENTS

L1 protein was purified from a crude leucocyte extract by preparative isoelectric focusing.¹ For analytical focusing, L1 samples (~50 μ g in 15 μ l water) were applied near the cathode of agarose gels containing 7.5% proprietary (LKB, Sweden), pH 5 to pH 8. The protein bands were transferred¹⁷ to a nitrocellulose sheet (Bio-Rad, USA), which was subsequently cut into two equal parts. These were soaked in appropriately diluted polyclonal or monoclonal antibody reagents (such as Mac 387 at 1/400) for three

hours at room temperature. Thereafter incubation took place overnight with peroxidase-labelled goat anti-rabbit or anti-mouse IgG conjugate at 1/1000 (Bio-Rad). The enzyme substrate was diaminobenzidine in 0.1 M sodium citrate, pH 5, with 0.015% H₂O₂.

Dot blot experiments were performed with crude leucocyte extract containing a known amount of L1 antigen and with two fractions of purified L1 protein.¹ After application of the samples (1 μ l) in two-fold serial dilutions (representing from 2000 to 0.2 ng L1 antigen), the nitrocellulose sheets were soaked in buffer containing BSA (30 g/l) overnight and were thereafter stained with the APAAP method using polyclonal anti-L1 or monoclonal Mac 387 applied as described above for ethanol-fixed tissue sections. Colostral IgA (2000 ng) was applied as a control protein sample, and monoclonal anti-IgA2 was used as a control murine antibody at a dilution comparable with that of Mac 387.

Results

IMMUNOHISTOCHEMISTRY

Mac 387 and polyclonal anti-L1 applied in the APAAP method produced virtually identical staining results, but Mac 387 required more appropriate trypsinisation of formalin fixed sections. In such sections more extracellular L1 was shown by the polyclonal reagent, probably because protein antigens are particularly masked in the interstitial matrix by cross linking fixatives.¹⁸

Both antibody reagents produced remarkably distinct staining of infiltrating macrophages (fig 1). Epithelioid cells were variably positive; macrophage giant cells were negative or only faintly stained. Various dendritic cells were generally negative as described previously.⁶ One exception was a minor population of putative interdigitating dendritic cells in some T cell areas of lymph nodes and tonsils; paired staining with Mac 387 and antiserum to S-100 protein showed a variable cell fraction with concomitant cytoplasmic staining (fig 2). This fraction could be somewhat increased in dermatopathic lymphadenopathy.¹⁹

Mac 387 and polyclonal anti-L1 both stained normal oral, pharyngeal, and vaginal squamous epithelium in a similar pattern; normal epidermis was negative except the pilosebaceous units.⁶

COMPARATIVE TWO-COLOUR STAINING

Sequential paired immunofluorescence, with Mac 387 applied before the polyclonal anti-L1, produced in all cases concordant staining features. Concomitant green and red cytoplasmic fluorescence was seen in granulocytes and histiocytes of reactive lymph nodes,

Fig 1 *L1-positive macrophages in adjacent sections of ethanol-fixed specimen from tonsillar non-Hodgkin's large cell lymphoma. APAAP labelling counterstained with haematoxylin. (a) Primary reagent was polyclonal antiserum to L1 antigen (b) primary reagent was monoclonal Mac 387.*

Fig 2 *Concurrent paired immunofluorescence staining of histiocytes in paracortical field from trypsinised section of formalin fixed reactive lymph node. Left panel: rhodamine labelling with antiserum to S-100 protein; right panel, fluorescein labelling with Mac 387. There are numerous S-100 protein-positive, presumably interdigitating dendritic cells (left), but also many cells of similar appearance detail labelled by Mac 387 (right); only few cells (arrowed) show concomitant labelling.*

Fig 3 Sequential paired immunofluorescence staining with anti-serum to L1 antigen (left panel, rhodamine) and Mac 387 (right panel, fluorescein) in field from a trypsinised section of formalin fixed specimen from a true histiocytic malignant lymphoma. All neoplastic cells show concomitant labelling.

Fig 4 Concurrent paired immunofluorescence staining with antiserum to L1 antigen (left panel, rhodamine) and Mac 387 (right panel, fluorescein) in field from a trypsinised section of formalin fixed specimen from a true histiocytic malignant lymphoma. Neoplastic cells show selective faint red staining, whereas granulocytes adjacent to vessel (V) show concomitant labelling.

Fig 5 Concurrent paired immunofluorescence staining with antiserum to L1 antigen (left panel, rhodamine) and Mac 387 (right panel, fluorescein) in a section of ethanol fixed specimen from ileal mucosa. All macrophages in Peyer's patch show concomitant labelling. V = villus epithelium; D = dome epithelium; LF = lymphoid follicle.

tonsils, and mucous membranes, and also in mucosal squamous epithelia. Two L1 positive histiocytic malignant lymphomas were likewise identically stained by the two reagents after sequential incubation (fig 3).

Conversely, when the two antibody reagents were mixed, concomitant fluorescence was mainly obtained where the L1 antigen was present in high concentrations, such as in granulocytes (fig 4) and squamous epithelia. With this approach Mac 387 failed to decorate weakly L1-positive subpopulations of normal or malignant (fig 4) histiocytes and extracellular L1 antigen; the polyclonal reagent presumably had a greater avidity for L1 and therefore partially blocked the reactivity of Mac 387.

Competitive inhibition of Mac 387 was not observed, however, when the mixed primary antibody reagents were applied on ethanol fixed sections, probably because the epitope detected by the monoclonal antibody was better preserved by this fixative. Thus in

such material there was always complete concordance between the two colours emitted by histiocytes subjected to paired staining (fig 5).

ABSORPTION WITH L1 ANTIGEN

When Mac 387 was absorbed with highly purified L1 antigen (0.1 g/l), the staining reaction was abolished for both leucocytes and epithelium. This held true both with formalin and ethanol fixed tonsillar sections. Only occasional granulocytes and epithelial cells with a high L1 concentration showed faint staining after application of absorbed Mac 387.

IMMUNOBLOTTING RESULTS

Mac 387 and polyclonal anti-L1 produced identical reaction patterns when compared in Western blots with purified L1 protein separated into various molecular species by isoelectric focusing (fig 6). In dot blots the polyclonal reagent produced a visible

Fig 6 Comparison of molecular patterns shown by Western blotting with Mac 387 (mono mac ab) or polyclonal antibody to L1 antigen (poly anti-L1). Purified L1 protein was subjected to isoelectric focusing in agarose gel on a pH 5 to pH 8 gradient, and the protein bands were transferred to a nitrocellulose sheet that was cut into two halves (left and right panel) before antibody incubation.

precipitate with crude leucocyte extract down to an L1 concentration of about 2 ng; the dots produced by Mac 387 were somewhat weaker and had an end point of about 8 ng. Parallel results were obtained with the two purified L1 fractions (fig 7). Such dots were not produced in the control sheets with colostral IgA or anti-IgA2. Altogether, the immunoblotting results excluded the possibility that Mac 387 reacted with an epitope that was unassociated with the L1 antigen.

Discussion

This study showed that the monocyte antibody Mac 387 reacts with the well defined human myelomonocytic L1 antigen. Our conclusion is based on physicochemical and immunohistochemical tests. It remains to be explained why the molecular weights reported for components detected by Mac 387 in detergent solubilised material obtained from granulocytes and monocytes¹¹ were somewhat inconsistent with those previously reported for L1.¹⁵ One possibility is that the Mac 387 antigens to some extent might have been

Fig 7 Dot blots obtained by testing polyclonal antibody to L1 antigen (poly anti-L1) or Mac 387 (mono mac ab) against two-fold dilutions (from top to bottom) of crude leucocyte extract (left lane in each panel) and two fractions of purified L1 antigen (middle and right lanes). TP indicates total amount of applied protein and LI indicates amount of specific antigen as determined by radioimmunoassay. Identical samples were applied in left and right panels, and the numbers show examples of the amounts of TP or L1 antigen in nanograms.

transmembrane L1 precursors released by the detergent. Moreover, without adequate enzyme inhibition, proteolysis readily takes place in leucocyte extracts. Another possibility is that L1 behaves differently according to the available concentration of calcium for which it has a remarkable affinity¹⁵; calcium may thus cause aggregation of L1 and its binding to other proteins. Such artefacts may not be equally expressed for detergent extracts of granulocytes and monocytes.

The specificity of Mac 387 for the well characterised L1 antigen^{13,5} renders this antibody a unique immunological reagent which is now commercially available (Dakopatts). Previous monoclonal antibodies to myelomonocytic markers have been raised against surface antigens and have been mainly defined by staining of cell suspensions. When further characterised by immunohistochemistry, these antibodies have performed satisfactorily only on cryostat sections. Moreover, they have shown striking and unexpected specificity heterogeneity, producing

virtually every possible combination of additional positive staining: T and B lymphocytes,^{20,21} natural killer cells,²² null cells,^{21,23} platelets,^{23,24} and a variety of dendritic cells.²⁵⁻²⁷ This is in contrast to the restricted leucocyte distribution of the L1 antigen.^{2,6} The monoclonal antibody 27E10 apparently showed reactivity with tissue macrophages and squamous epithelium similarly to Mac 387, but it was reported to detect a 17 kD protein and was rarely seen in granulocytes of inflammatory lesions.²⁸ Conversely, A1-3 seemed to react with tissue granulocytes and macrophages similarly to Mac 387, but it was reported to detect a 52 kD protein and cross reacted with some fibroblasts and secretory epithelia.²⁹

Unexpected cross reactions—for example, with fibroblasts, endothelium, epithelium, and melanoma cells—have, in fact, been observed by several authors who have tested monoclonal antibodies to myelomonocytic antigens by immunohistochemistry.^{22,28-35} Such results, however, might be due to common or partially identical epitopes on different molecules.³⁶ To our knowledge L1 is the first protein in addition to HLA molecules and interleukin 1,³⁷ directly shown to be expressed by both squamous epithelium and leucocytes.⁷ The epithelial reactivity of Mac 387 therefore has a defined molecular explanation. Because of the apparent structural identity of the cystic fibrosis antigen⁴ with the L1 protein,³ epithelial reactivity for the former⁴ likewise reflects true antigen expression by keratinocytes.

As L1 is a formalin resistant marker of both macrophages and squamous epithelium, Mac 387 may have several immunohistochemical applications on routine pathological material. (a) L1 is excellent for labelling of infiltrating histiocytes in malignant lymphomas, thereby identifying cells that may confuse the morphological interpretation by mimicking the neoplastic population.^{6,11} (b) By paired staining for L1 and S-100 protein¹⁹ or HLA-DR,⁶ the proportion of two types of reactive histiocytes can be determined; there are some data to indicate that such information is of prognostic value in relation to certain neoplasms.³⁸⁻⁴⁰ (c) L1 is a useful marker of some truly histiocytic tumours (figs 3-4) it has been difficult to identify all such malignancies by previously available phenotypic characteristics.^{10,41,42} (d) L1 is an interesting epidermal marker in a variety of inflammatory skin diseases⁸ and skin tumours.⁹ (e) L1 is a promising marker for distinction between squamous cell carcinoma and other carcinomas of the lung.⁴³ The availability of a monoclonal antibody to L1 will hopefully facilitate further immunohistochemical studies of this interesting cell marker protein.

This work was supported by grants from the Norwegian Cancer Society, The Norwegian Society for

Fighting Cancer, the Norwegian Research Council for Science and the Humanities, and the Anders Jahre Fund.

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Problem Solving in Immunohematology. 3rd ed. Herbert Silver. (Pp 123; \$30.) Raven Press. 1988. ISBN 0 89189 263 X.

This excellent short book has an entertaining way of presenting a wide range of problems in blood banking. Its chapters cover processing of donor blood, tests on prospective recipients, antibody identification, blood component inventories, transfusion reactions, prenatal and neonatal testing, Rh immunoglobulin, diagnosis of HDN, and selection of blood for exchange transfusion. The only omission seems to be autoimmune haemolysis. Each section provides a surprising amount of information: a short introduction is followed by a series of exercises aimed to cover the major problems likely to arise in that area. The reader is invited to solve the problems before turning to the authors' answers and interpretations. The book is intended to develop a methodical approach to blood bank problems. It succeeds admirably, is fun to use, and will be of value to both clinical and laboratory haematologists. I thoroughly recommend it.

RJ SKOL

Campylobacter Pylori. Ed H Menge, M Gregor, GNJ Tytgat, BJ Marshall. (Pp 249; £30.) Springer. 1988. ISBN 3 540 18761 8.

The proceedings of a symposium on a single organism sounds esoteric. This one is not. It is a collection of papers and discussions by a select group of gastroenterologists, microbiologists, histopathologists, and physiologists on the subject of gastritis and peptic ulcer disease looked at in the light of the recent discovery of *Campylobacter pylori*. This interdisciplinary approach gives a welcome breadth to the subject. Many of the 26 papers include informative reviews, for example, on current theories of the pathogenesis and medical management of peptic ulcer disease, and the cytoprotective action of bismuth salts. The book is well produced, with good photographic reproductions. There are a few phonetic misspellings of names mentioned in the discussions but otherwise the text seems to be accurate.

Books of this sort tend to go out of date quickly, but this one gives sufficient grounding in the subject to maintain its interest for many. Like most symposium reports it is rather expensive.

MB SKIRROW

Notices

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The fee is £195.00 (registration *before* 9 June 1989) and £225.00 after that date. Further details from: Mrs L Barducci, British Association of Dermatologists, 6 St Andrew's Place, Regent's Park, London, NW1 4LB (tel 01 935 8576).

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Forms for this purpose and further information can be obtained from Mrs J Hopkins, 7th Floor Haematology Laboratory, University of Wales College of Medicine, Heath Park, Cardiff CF4 4XN.

The Leeds Course in Clinical Nutrition

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Further details from: Mrs H L Helme, Department of Continued Professional Education, The University, Leeds LS2 9JT.

Pathological Effects of Radiation

11-13 September 1989 at the Holiday Inn of Bethesda, Bethesda, Maryland

For further information contact: David Busch, American Registry of Pathology, Armed Forces Institute of Pathology, Washington, DC 20306-6000.

Correction

Errors were made in the text of the paper by Brandtzaeg *et al* *J Clin Pathol* 1988;41:963-70. In the Material and methods section under the heading of *Immunoblotting Experiments*, the second sentence should have read: For analytical focusing, L1 samples (~50 µg in 15 µl water) were applied near the cathode of agarose gels containing 7.5% Ampholine (LKB, Sweden), pH5 to pH8. The legend to figure 2 should have contained the following: There are numerous S-100 protein positive, presumably interdigitating dendritic cells (left), but also many cells of similar appearance labelled by Mac 387 (right). He apologises for these oversights.