In Monoclonal antibodies and T cell hybridomas. U. Hammerling, G. Hammerling, and J. Kearney, editors. Elsevier, New York. 53-61. 1981.

1.2.5. Rat anti-mouse macrophage monoclonal antibodies and their use in immunofluorescent studies of macrophages in tissue sections

MAY-KIN HO and TIMOTHY A. SPRINGER

Laboratories of Membrane Immunochemistry, Sidney Farber Cancer Institute, Harvard Medical School, Boston (Massachusetts, U.S.A.)

Introduction

There is increasing evidence indicating that macrophages play an important role in various immune responses (reviewed in ref. 1). However, the study and isolation of these cells were hampered by the lack of specific antibodies to macrophage antigens. Previously, several workers have obtained anti-macrophage sera by xeno- or alloimmunization followed by tissue absorption [2–6]. Some of these sera were found to inhibit the adherent and phagocytic properties of macrophages. However, their use as probes of structure–function relationships was limited because antibodies to more than one antigenic species were usually present in these antisera. To circumvent this problem of multiple and sometimes variable specificities of conventional anti-macrophage reagents, we have produced monoclonal antibodies (MAb) to macrophage antigens. In this communication, we summarize the characteristics of five such MAb, and describe the use of one of these MAb for the localization of macrophages in frozen spleen sections.

Methods

Cell lines

Rat spleen cells immunized to mouse spleen cells (M1 clones) [7,8] or thioglycollate-elicited peritoneal exudate cell glycoproteins depleted of previously recognized antigens with MAb immunoabsorbents (M3 clones) [9] were fused with the mouse

myeloma NSI as described[10]. Hybridomas used here have been subcloned at least twice. Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10 % horse serum or 5 % fetal calf serum and the spent culture supernatants used as a source of MAb.

Immunofluorescent staining of spleen sections

Fresh spleens from male BALB/c mice were frozen in OCT compound (Ames Co., Div. of Miles Laboratories, Elkhart, IN). Three μm sections were cut and stored at –35°C for up to two weeks. Sections were thawed, fixed in acetone for 15 min, and washed twice in PBS. Subsequently, they were stained with 40 μl of anti-Mac-1 (M1/70, 5 μg/ml); or anti-Thy-1 (M5/49[11], 5 μg/ml) supernatants for 30 min, washed twice in phosphate-buffered saline (PBS), and stained for another 30 min with 40 μl of fluorescein isothiocyanate (FITC) $F(ab)'_2$ rabbit anti-rat IgG previously absorbed with mouse IgG. All staining was performed at room temperature in a moist chamber. After washing twice in PBS, the sections were mounted in Aqua-mount (Lerner Laboratories, Stamford, CT). Controls include sections stained with anti-Mac-1, or anti-Thy-1 MAb only, as well as with NSI clonal supernatants plus 5 μg/ml normal rat IgG followed by the second stage FITC reagent.

Results

Characteristics of monoclonal antibodies to mouse macrophage antigens

Five rat monoclonal antibodies to mouse macrophage surface antigens have been developed in our laboratory by the myeloma–spleen cell hybrid technique of Kohler and Milstein[10] (Table 1). M1/70, which recognizes a phagocyte-specific antigen, Mac-1, is the most extensively studied[8]. Mac-1 is composed of two polypeptides of $M_r = 190{,}000$ and 105,000. Immunofluorescence flow cytometry and ^{125}I anti-rat IgG indirect binding studies show that Mac-1 is expressed abundantly on thioglycollate-induced macrophages and, in lesser quantities, on blood monocytes, granulocytes, and subpopulations of cells in bone marrow or spleen. M1/70 also cross-reacts with monocytes, granulocytes, and natural killer cells in human peripheral blood[13].

Two other antibodies, M3/31 and M3/38, precipitate a $M_r = 32{,}000$ and lesser amounts of a $M_r = 30{,}000$ polypeptide, termed Mac-2. Antigenic determinants recognized by M3/31 and M3/38 are on the same molecular species because precipitation with M3/31 removes material recognized by M3/38 and vice versa. Mac-2 is synthesized by macrophages because the same polypeptides are labeled with $[^{35}S]$-methionine in purified cells. In contrast to Mac-1, Mac-2 is not found on bone

TABLE 1

Monoclonal rat anti-mouse macrophage antibodies

Antibody	Subclass	Antigen	Polypeptide chains (M_r)	Distribution[a]		Reference
				Positive	Negative	
M1/70	IgG2b	Mac-1	190,000 105,000	(PP, LM, LPS, Con A, TG)-PEM, PM, SM, M, G, 50% BM	T, PLN	8 12
M3/31 M3/38	IgM IgG2a	Mac-2	32,000	TG-PEM 20% PP-PEM[b]	>97% (LM, LPS, Con A)-PEM, PM, SM, G, T, PLN, >99% BM	9, 10 12
M3/84	IgG1	Mac-3	110,000	TG-PEM	T, PLN, >99% BM	9, 10, 27
M3/37[c]	?	Mac-4	180,000	TG-PEM	T, >99% BM	9, 10, 27

[a] Abbreviations used: PEM, peritoneal exudate macrophages; PM, unelicited peritoneal macrophages; SM, splenic macrophages; M, monocytes; G, granulocytes; BM, bone marrow cells; T, thymus cells; PLN, peripheral lymph node cells; TG, thioglycollate; PP, protease peptone; LM, L-monocytogenes; LPS, lipopolysaccharide; Con A, concanavalin A.

[b] Weakly positive.

[c] Not cloned.

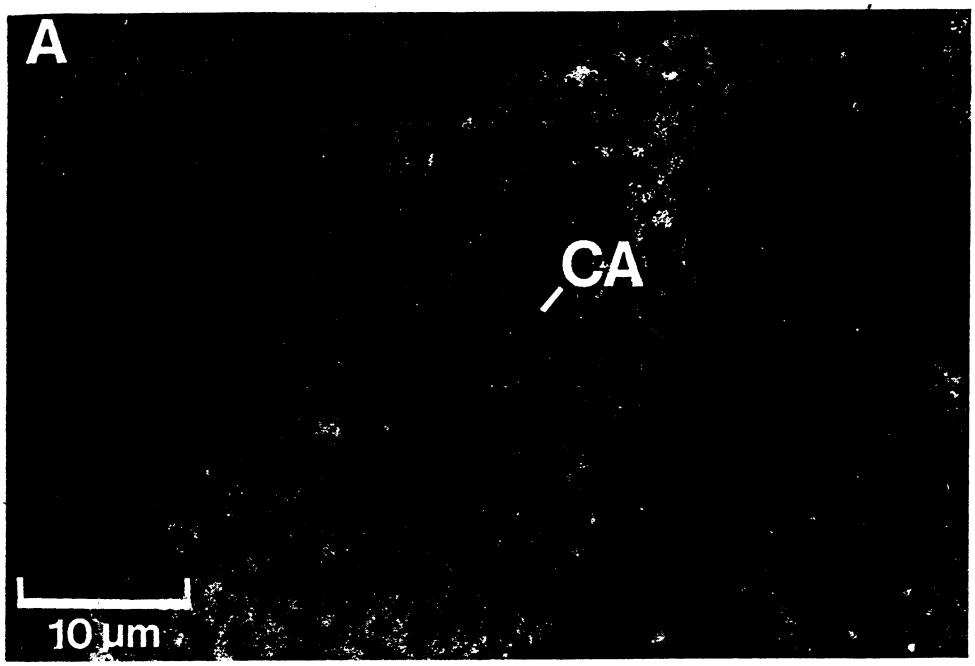
marrow cells, granulocytes, or spleen cells. Moreover, preliminary studies indicate that Mac-1 is expressed on unelicited peritoneal macrophages as well as macrophages induced by thioglycollate, peptone, concanavalin A, LPS, and *Listeria monocytogenes*. However, Mac-2 is strongly expressed on only thioglycollate-elicited cells. Twenty percent of macrophages induced by peptone are weakly positive whereas > 97 % of cells induced by other means are negative. Therefore, Mac-2 seems to be associated with only certain macrophage subpopulations.

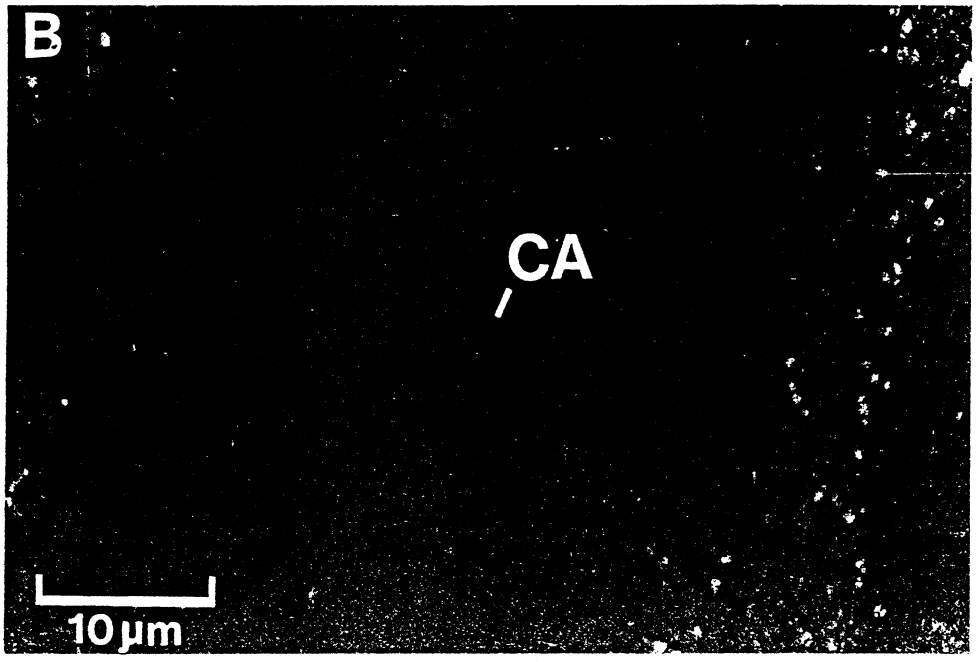
M3/84 (anti-Mac-3) and M3/37 (anti-Mac-4) identify antigens of $M_r = 110{,}000$ and 180,000 respectively. These antigens are expressed by thioglycollate-elicited macrophages but not lymphoid or bone marrow cells.

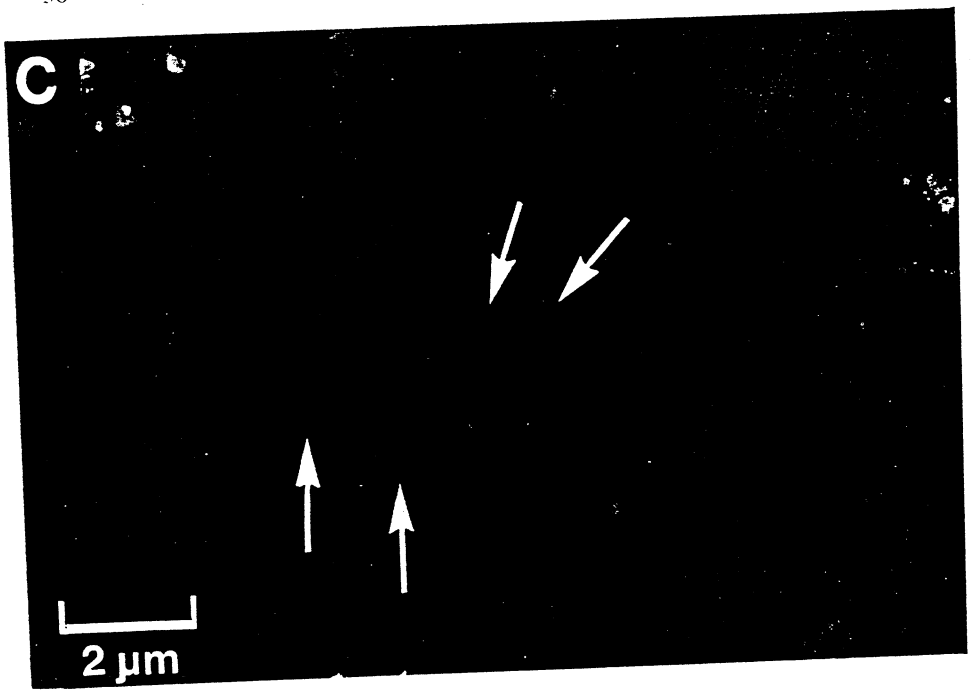
Use of anti-Mac-1 to stain macrophages in spleen sections

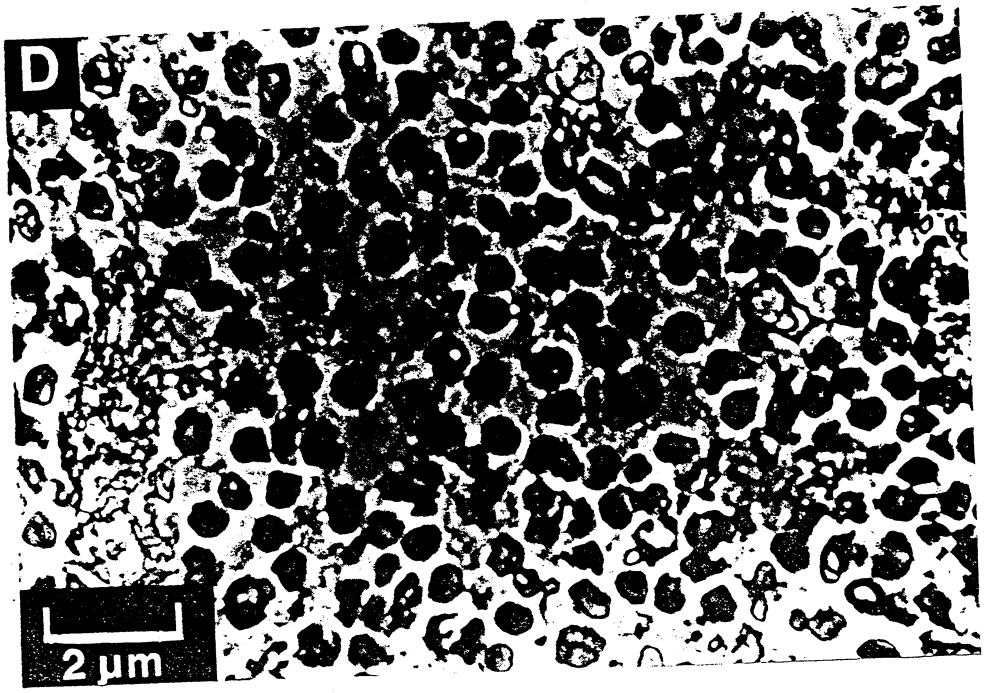
The fine anatomical localization of lymphocyte subpopulations has been examined in detail by both histological, autoradiographic, and immunofluorescent methods[14–17]. In contrast, few immunofluorescent studies of tissue macrophages have been performed due to the lack of specific antibodies to macrophage antigens. This prompted us to employ our antibodies for the study of macrophages in frozen spleen sections by indirect immunofluorescence. Anti-Mac-1 was chosen for the studies reported here because Mac-1 seems to be expressed on macrophages irrespective of their state of differentiation and activation. To allow alignment of areas with Mac-1$^+$ cells with T-dependent areas of the spleen, adjacent sections were stained with M5/49, an anti-Thy-1 MAb[11]. As seen in Fig. 1A, T lymphocytes in the periarteriolar lymphatic sheath are intensely stained by anti-Thy-1 MAb. In contrast, few, if any, Mac-1$^+$ cells can be seen in these T-dependent areas. However, numerous highly Mac-1 positive cells are found in the marginal zone (Fig. 1B) and red pulp (Fig. 1C). Occasionally, some stained cells are also seen in the T-independent area of white pulp. Two-color immunofluorescence staining with anti-Mac-1 and anti-Thy-1 reagents have also been carried out in single sections (result not shown); similar results were obtained. Since the morphology of cells in frozen sections is not well preserved, we also stained adjacent sections with hematoxylin and eosin. Most of the cells stained strongly by anti-Mac-1 do appear to have the morphological and staining characteristics of macrophages. In addition, there exist some smaller cells with segmented nuclei, mostly in the red pulp, which are stained

Fig. 1. Immunofluorescent staining of spleen sections with anti-Thy-1 and anti-Mac-1 MAb. Frozen spleen sections were stained anti-Thy-1 (A) or anti-Mac-1 (B,C) followed by FITC-F(ab')$_2$ rabbit anti-rat IgG. Thy-1$^+$ cells are located in the periarteriolar lymphatic sheath (A), whereas Mac-1$^+$ cells are scattered in the marginal zone (B) (adjacent sections). A branch of the central artery (CA) can be seen in both sections. Mac-1$^+$ cells (arrows) are also found in the red pulp (C). D is the same field viewed under phase contrast.


A
CA
10 µm


B
CA
10 µm


C
2 µm


D
2 µm

weakly for Mac-1. They probably represent granulocytes which have previously been shown to express only small quantities of Mac-1 [8].

A few similar experiments have also been carried out with anti-Mac-2. Preliminary results indicate that < 1 % of cells in normal spleen are weakly stained. This is in accordance with the finding that Mac-2 expression is restricted to distinct macrophage subpopulations such as thioglycollate-elicited cells. Whether Mac-2 is expressed on splenic macrophages after antigenic stimulation in vivo has yet to be investigated.

Discussion

To date, mononuclear phagocytes in tissue sections are usually localized by their morphology, phagocytic or pinocytic ability, and enzyme markers [18–21]. However, morphological characteristics are not always sufficient for the identification of these cells. Administration of particles in vivo allows the localization of macrophages on the basis of both morphology and their ability to take up these particles. This approach can be time-consuming and laborious, especially if electron microscopy is involved. Moreover, administration of particles to neonatal animals can be technically difficult. Enzymes, such as non-specific esterase, peroxidase, and lysozyme, can be used as macrophage markers only in certain cases, because the quantities of some of these enzymes are dependent on the stage of maturation. Therefore, the method described here is a simple alternative for the localization of tissue macrophages. Because the accuracy of this procedure depends largely on the specificity of the antibodies used, MAb are the reagents of choice. Even though Mac-1 is not exclusively associated with macrophages, it can be used as a general macrophage marker. This is because the staining intensity, size, and morphology of granulocytes are sufficiently different from that of macrophages so that the latter can be identified with ease.

It is interesting to note that glass-adherent cells isolated from single spleen cell suspensions are only weakly stained by M1/70 (unpublished results), whereas macrophages in spleen sections show intense staining. We usually obtain spleen cells by manually teasing spleens with forceps, allowing residual tissue fragments to settle, and then recovering single cells from the supernatant. Steinman and Cohn [22] observed that most splenic glass-adherent cells were monocytes when isolated by similar procedures. Moreover, large numbers of macrophages can be recovered by collagenase treatment of big tissue clumps which are normally discarded in such preparations [22]. Therefore, it is likely that the adherent cells we examined were mostly monocytes which have been shown to express lesser amounts of Mac-1 [8]. In addition to macrophages, collagenase treatment of spleen also yields a small percentage of dendritic cells which have morphological and phagocytic properties distinct

from macrophages[22]. Recent studies indicate that these cells are potent stimulators of mixed lymphocyte reactions[23,24] and are accessory cells for the development of anti-trinitrophenyl cytotoxic T lymphocytes[25]. Whether dendritic cells are related to macrophages is not clear. However, in the light of the localization of dendritic cells in splenic white pulp[26] and the scarcity of Mac-1$^+$ cells in this region, dendritic cells are probably not stained by anti-Mac-1.

The simple method described in this publication can be easily extended to other studies, especially with anti-Mac-1, which stains all macrophage subpopulations examined so far. Some examples of these studies are: 1) macrophage localization after antigenic stimulation or infection by bacteria and parasites; 2) distribution of macrophages in various tissues, such as lung, liver and thymus; 3) examination of granulomas which may contain mononuclear phagocytes at different stages of differentiation, namely, monocytes, macrophages, epithelioid cells, and multinucleate giant cells; and 4) macrophage development in various tissues during ontogeny. In view of the vast body of information gained from anatomical localization of lymphocyte subpopulations, these studies should provide much insight into the function, differentiation, and ontogeny of macrophages.

Acknowledgement

This work was supported by USPHS grant CA 31799 and by the Council for Tobacco Research. M.-K. Ho is a postdoctoral fellow of the Anna Fuller Fund.

References

1 Nelson, D. S. (ed.), 1976, Immunobiology of the Macrophage. Academic Press, New York.
2 Unanue, E. R., 1968, Nature 218, 36.
3 Stinnett, J. D., A. M. Kaplan and P. S. Morahan, 1976, J. Immunol. 116, 273.
4 Gallily, R. and M. Gornostansky, 1972, Immunology, 22, 431.
5 Archer, J. R. and D. A. L. Davies, 1974, J. Immunogen. 1, 113.
6 Tiller, G. E., A. R. McGiven and E. Pihl, 1973, Aust. J. Exp. Biol. Med. Sci. 51, 793.
7 Springer, T., G. Galfre, D. S. Secher and C. Milstein, 1978, Eur. J. Immunol. 8, 539.
8 Springer, T., G. Galfre, D. S. Secher and C. Milstein, 1979, Eur. J. Immunol. 9, 301.
9 Springer, T., 1981, J. Biol. Chem. 256, 3833.
10 Springer, T. A., 1980, in: Monoclonal Antibodies, eds. R.H. Kennett, T. J. McKearn and K. B. Bechtol (Plenum, New York) p. 185.
11 Davignon, D., E. Martz, T. Reynolds, K. Kürzinger and T. A. Springer, 1981, Proc. Natl. Acad. Sci. U.S.A. 78 (in press).
12 Ho, M.K. and T. Springer, 1981, Manuscript in preparation.
13 Ault, K. A. and T. A. Springer, 1980, J. Immunol. 126, 359.
14 Weiss, L., 1972, in: The Cells and Tissues of the Immune Response. Structure, Functions, Interactions, eds. E.G. Osler and L. Weiss (Prentice Hall, Englewood Cliffs, NJ).
15 Van Ewijk, W., J. Rozing, N. H. C. Brons and D. Klepper, 1977, Cell Tiss. Res. 183, 471.

16 De Sousa, M.A.B. and D.M.V. Parrott, 1967, in: Germinal Centers in Immune Responses, eds. H. Cottier, N. Odartchenko, R. Schindler and C.C. Congdon (Springer-Verlag, Berlin) p. 361.
17 Ledbetter, J. A., R. V. Rouse, H. S. Micklem and L. A. Herzenberg, 1980, J. Exp. Med. 152, 280.
18 Hoefsmit, E. C. M., 1973, in: Mononuclear Phagocytes in Immunity, Infection and Pathology, ed. R. van Furth (Blackwell Scientific Publications, Oxford) p. 129.
19 Chen, L. L., A. M. Frank, J. C. Adams and R. M. Steinman, 1978, J. Cell Biol. 79, 184.
20 Veerman, A. J. P. and W. Van Ewijk, 1975, Cell Tiss. Res. 156, 417.
21 Nossal, G. J. V., A. Abbot, J. Mitchell and Z. Lummus, 1968, J. Exp. Med. 127, 277.
22 Steinman, R. M. and Z. A. Cohn, 1973, J. Exp. Med. 137, 1142.
23 Nussenzweig, M. C. and R. M. Steinman, 1980, J. Exp. Med. 151, 1196.
24 Steinman, R. M. and M. D. Witmer, 1978, Proc. Natl. Acad. Sci. U.S.A. 75, 5132.
25 Nussenzweig, M. C., R. M. Steinman, B. Gutchinov and Z. A. Cohn, 1980, J. Exp. Med. 152, 1070.
26 Steinman, R. M., J. C. Adams and Z. A. Cohn, 1975, J. Exp. Med. 141, 804.
27 Springer, T. A., 1981, in: Heterogeneity of mononuclear phagocytes, ed. O. Forster (Academic Press, New York) (in press).