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Abstract: In order to characterize in situ the macrophages present in experimental pyogranulomas induced in lambs with *Corynebacterium pseudotuberculosis*, a set of monoclonal antibodies (MAbs) was produced following immunization of BALB/c mice with alveolar macrophages from healthy sheep. Three MAbs were retained after two steps of screening using alveolar macrophages, peripheral blood lymphocytes, and polymorphonuclear leukocytes as target cells. Their reactivity was tested not only on macrophages in pyogranulomas but also on sections of various organs in steady-state conditions. One MAb, termed OM1, recognized the monocytes and the majority of cells of the mononuclear phagocyte system in lymphoid and nonlymphoid organs. The two other MAbs, OM2 and OM3, reacted with a subpopulation of alveolar macrophages and with other cell types in tissues, in particular with endothelial cells for the MAb OM2. On sections of experimental pyogranulomas that developed in lymph nodes draining the *C. pseudotuberculosis*-injected sites, MAb OM1 reacted with all the macrophages distributed in a palisade surrounding the necrotic center of the lesion from day 6 to day 28 postinoculation. The two other MAbs, OM2 and OM3, enabled two types of granulomas to be distinguished: one type was characterized by a large number of epithelioid cells stained by OM2; and the other was characterized by a few OM2-positive macrophages, whereas the OM3-positive cells were more numerous. These results show that macrophages are predominant cells in pyogranulomas and suggest two different histological patterns in the evolution of pyogranulomas induced by *C. pseudotuberculosis*, according to the immunological status of the host. *J. Leukoc. Biol.* 51: 188-198; 1992.

Key Words: *macrophage* • *monoclonal antibodies* • *immunohistochemistry* • *pyogranuloma* • Corynebacterium pseudotuberculosis • *sheep*

INTRODUCTION

From all hematopoietic stem cell-derived lineages, mononuclear phagocytes constitute the most heterogeneous cell population. These cells arise in the bone marrow, pass briefly through the blood as monocytes, and then immigrate into every tissue, where they may express different phenotypical and functional characteristics according to their localization in the body and their state of activation [20, 54]. Macrophages, whether in steady-state conditions or activated by cellular immune mechanisms, are central cells involved in host defense against intracellular bacterial pathogens [22], especially in granulomatous lesions induced by these pathogens [1, 17, 57]. In order to study the interactions between macrophages and intracellular bacteria, we have developed an ovine model in which infectious pyogranulomas can be successfully and consistently produced in lymph nodes as well as in the lungs following subcutaneous inoculation of viable *Corynebacterium pseudotuberculosis* into the dorsum of ear [39, 40]. *C. pseudotuberculosis*, a gram-positive bacillus, is the causative agent of caseous lymphadenitis in sheep and goats [6]. In previous studies, we have shown that the recruitment of polymorphonuclear leukocytes is important only during the early phases of infection [21], and we have hypothesized that mononuclear phagocytes should dominate the sites resulting from the transformation of the primary foci into typical pyogranulomas. Morphological studies of these pyogranulomas showed that four different parts could be described in a typical lesion: a necrotic center, two cellular layers composed respectively of mononuclear phagocytes and lymphocytes, and a fibrous wall [21, 38]. To check the presence of macrophages in the pyogranulomatous lesions, a set of monoclonal antibodies (MAbs) against ovine macrophages was produced.

In the present study, *C. pseudotuberculosis*-induced pyogranulomatous lesions were investigated immunohistochemically with three murine MAbs raised against ovine macrophages. These MAbs were obtained after immunization with ovine alveolar macrophages. Their reactivities were compared in different tissues and in pyogranulomas at various stages of development. One MAb, OM1, recognized the majority of macrophages in lymphoid and nonlymphoid organs and labeled all the macrophages distributed in a palisade surrounding the necrotic center of pyogranulomas. The two other MAbs, OM2 and OM3, which recognized other cell types, reacted with subsets of macrophages infiltrating the pyogranulomas.

Abbreviations: APAAP, complex of alkaline phosphatase and monoclonal mouse anti-alkaline phosphatase; ELISA, enzyme-linked immunosorbent assay; Ig, immunoglobulin; MAb, monoclonal antibody; MHC, major histocompatibility complex; PBS, phosphate-buffered saline; PI, postinoculation; PMN, polymorphonuclear leukocyte; PNPP, *p*-nitrophenyl phosphate; TBS, Tris-buffered saline.

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MATERIALS AND METHODS

Animals and Experimental Infections

Experimental pyogranulomas were induced in 3-month-old male Préalpes du Sud lambs inoculated subcutaneously in the dorsum of the right ear with 1.8–3.0 × 10^8 viable bacteria of *C. pseudotuberculosis*, strain 19R [39, 40]. Prior to experimental infection, lambs were verified to be free from clinical abscesses and were negative for antibodies against *C. pseudotuberculosis* exotoxin [40]. Growth, storage, and preparation of strain 19R for inoculum procedures were as previously described [37].

Cells and normal tissues were obtained from healthy adult sheep.

BALB/c mice (Iffa-Credo. L'Arbresle, France) were used for immunization with ovine alveolar macrophages and production of ascites.

Histochemical Reagents and Antibodies

Naphthol AS-TR phosphate (No. N-6000), Fast Red TR salt (No. F-1500), levamisole (No. L-9756), *p*-nitrophenyl phosphate, disodium (PNPP; No. 104-105), naphthol AS-BI phosphate (sodium salt; No. N-2250), Fast Red Violet LB salt (No. F-3381), glycerol gelatin (No. GG-1), hematoxylin solution (No. HHS-1-16), and mounting medium (No. 1000-4) were obtained from Sigma Chemical Co. (St. Louis, MO). Rabbit immunoglobulins (Ig's) to mouse Ig (No. Z259), complex of alkaline phosphatase and monoclonal mouse anti-alkaline phosphatase (APAAP; No. D651) were obtained from Dakopatts S/A (Glostrup, Denmark). Fluorescein-conjugated goat $F(ab')_2$ anti-mouse IgG was obtained from Cappel (No. 1311-0085, Malvern, PA).

An MAb obtained from Melbourne University (Australia) and raised against sheep major histocompatibility complex (MHC) class II molecules was used [43].

Obtention of Alveolar Macrophages and Blood Polymorphonuclear Leukocytes and Lymphocytes

Alveolar macrophages were harvested from healthy sheep by bronchoalveolar lavage as described by Charley et al. [9]. Immediately after slaughtering, a plastic tube was passed via the trachea to one lobe of isolated lung. A total of 180–240 ml of sterile phosphate-buffered saline (PBS; pH 7.2) minus Ca^{2+} and Mg^{2+} (PBS-A) was introduced in 60-ml aliquots. The lobe was gently massaged and the lung washing was collected into a sterile glass bottle on ice. The same procedure was repeated for other lobes. Thereafter, the lung washings were filtered on sterile gauze to remove mucus strands and then centrifuged at 250 *g* for 30 min to separate the cells. The supernatant fluid was removed and, if necessary, red blood cells were lysed by suspending the cell pellet for 45–60 s in sterile distilled water. Lysis was stopped by adding PBS-NaCl 2.7%. The cells were washed twice with PBS-A and resuspended at a concentration of 2 × 10^7 cells/ml in PBS. The number and the viability of cell suspension were determined by the trypan blue exclusion test. Viability was always greater than 80%.

Polymorphonuclear leukocytes (PMNs) and lymphocytes were obtained from blood of healthy sheep as already described [7, 21, 35]. Percentages of PMNs or lymphocytes in these cell suspensions were always greater than 85% with a viability greater than 95%. Cells were finally resuspended at a concentration of 10^8/ml in PBS.

Alveolar macrophages, PMNs, or lymphocytes were used either to obtain cytospin preparations (2 × 10^5 cells per slide fixed in acetone at 20°C for 10 min and stored at −70°C before use) or to coat 96-well plates for the cell-enzyme-linked immunosorbent assay (cell-ELISA) technique (see below). Alveolar macrophages were also used for immunization of BALB/c mice.

Monoclonal Antibody Production

Female BALB/c mice aged 8–10 weeks were immunized intraperitoneally with 10^7 alveolar macrophages suspended in 0.5 ml of PBS. This was repeated three times at 3-week intervals. Fusion of the mouse spleen cells and SP2/0 myeloma was performed as described elsewhere [18]. Briefly, 3 days after the last booster injection performed via the intravenous route, splenic cells of mice were fused with an equal number of cells of the nonsecreting myeloma cell line X63-Ag8.653. Fused cells were distributed into 24-well fusion plates (Becton Dickinson, Meylan, France) at a concentration of 1 × 10^6 cells/well using 1 × 10^5 BALB/c thymocytes per well as feeder layer. Screening of the supernatants was performed in two steps: first by using a cell-ELISA technique for a rapid screening (see below) and thereafter by a three-step immunoalkaline phosphatase stain on different cell suspensions (alveolar macrophages, PMNs, or lymphocytes) or on cryostat sections of ovine lymph nodes (see below). Selected hybridoma colonies were subjected to limiting dilution procedures, processed for mass culturing, and harvested as ascites from incomplete Freund's adjuvant-primed (Difco, Detroit, MI) BALB/c mice. Monoclonality and the isotype of selected antibodies were determined by hemagglutination with a commercial kit (Serotec-Realef, Paris, France).

Cell-ELISA for the Selection of Monoclonal Antibodies

A cell-ELISA technique as described by Baumgarten [5] was adapted for the first screening of antibodies. Falcon 96-well culture plates (AES, Combourg, France) were coated with alveolar macrophages, PMNs, or lymphocytes at a concentration of 3 × 10^5 cells/well in PBS. Plates were centrifugated at 700 *g* for 2 min. Supernatant was discarded and cells were fixed in cold 80% acetone (200 µl/well) for 10 min, air dried, and stored at −70°C before use. After thawing at 20°C, plates were gently washed twice in 0.05 M Tris-buffered saline (TBS; 200 µl/well), pH 7.6, with 0.0025% levamisole for inhibition of endogenous alkaline phosphatase for 15 min. Thereafter, they were incubated for 45 min with culture supernatant (100 µl/well) at 37°C. After washing in TBS, plates were covered with a 1:30 dilution of rabbit anti-mouse Ig (Dako; 70 µl/well) in TBS for 45 min at 37°C. Plates were washed twice again in TBS and incubated with a 1:60 dilution of APAAP complex (Dako) in TBS for 45 min at 37°C. After washing in 0.05 M Tris-HCl buffer, pH 8.2, immune alkaline phosphatase was visualized by adding 100 µl/well of substrate (5 mg PNPP, 500 µl Tris-HCl buffer, pH 8.2, and 4.5 ml 1.5 M NaCl). After incubation for 1 h at 37°C, plates were read at 410 nm. Controls were incubated in the same way, omitting the first step, or using a negative serum obtained from naive BALB/c mice before immunization.

Immunofluorescent Labeling

Positive culture supernatants were tested on live alveolar macrophage, granulocyte, or lymphocyte suspensions by indirect immunofluorescence [28]. Cells were incubated with supernatants for 45 min at 4°C followed by an incubation with a fluorescein-conjugated goat $F(ab')_2$ anti-mouse IgG (Cappel) for 45 min at 4°C. After three washes in PBS, cells

were then resuspended with a few drops of mounting medium and examined with an epifluorescence microscope (Leitz Aristoplan, Wetzlar, Germany).

Immunohistochemistry and Enzyme Histochemistry

The specificity of positive culture supernatants in cell-ELISA was characterized by immunocytochemistry on various cell populations. The selected MAbs were then tested on various lymphoid and nonlymphoid organs of healthy sheep and on ovine lymph nodes where the *C. pseudotuberculosis*-induced pyogranulomas developed.

Immediately after necropsy of carcasses of healthy adult sheep or experimentally infected lambs, pieces of various tissues or infected lymph nodes were removed and mounted in embedding compound (OCT, Tissue Tek; Miles, Elkhart, KS USA) on aluminum stubs. These samples were slowly immersed in liquid nitrogen and thereafter stored at −70°C before use. Sections 5–7 μm thick were cut in a Leitz cryostat (Wetzlar, Germany), placed on clean slides, air dried overnight, and fixed in acetone at 20°C for 10 min. Slides were wrapped in aluminum foil and stored at −70°C before use.

After thawing, cryostat sections or, in the case of cell suspensions, cytospin preparations were fixed again in acetone and thereafter rehydrated for 15 min with TBS, pH 7.6. Sections were incubated with culture supernatant for 45 min in a humid chamber. After three washes in TBS, the immunohistochemistry procedure was performed as described by Cordell et al. [11] by using the same steps and reagents described for the cell-ELISA (see above) except for visualizing the immune alkaline phosphatase. After washing in TBS, pH 8.2, the slides were stained with the filtered substrate (20 mg naphthol AS-TR phosphate, 2 ml dimethylformamide, 30 μg levamisole, 100 ml 0.1 M TBS, pH 8.2, and 100 mg Fast Red TR salt) for 20 min. Sections were washed in distilled water for 10 min, counterstained with hematoxylin, and mounted in glycerol gelatin.

Acid phosphatase activity was demonstrated as described by Dannenberg and Suga [13]. Briefly, the cell smears or frozen tissue sections, fixed in acetone, were incubated with the filtered substrate (10 mg naphthol AS-BI phosphate, 1 ml dimethylformamide, 100 ml 0.1 M acetate buffer, pH 5.2, 20 mg Fast Red Violet LB salt, 50 μl 10% $MnCl_2$) at 20°C for 60 min. After incubation, the rinsing, counterstaining, and mounting procedures were the same as for immunohistochemistry.

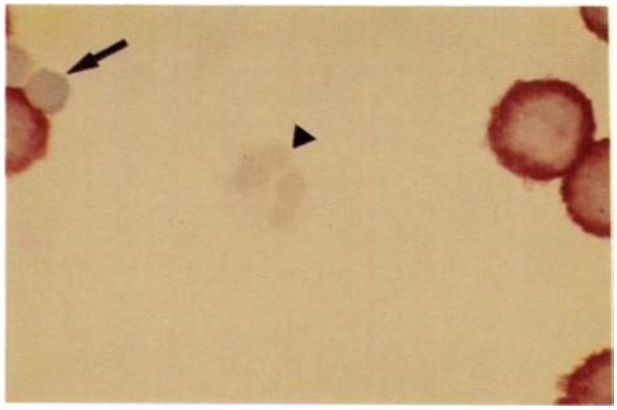


Fig. 1. Immunocytochemical staining with MAb OM1 on ovine alveolar macrophages. Note the presence of neutrophils (arrowhead) and lymphocytes (arrow), which are negative. [×500; APAAP].

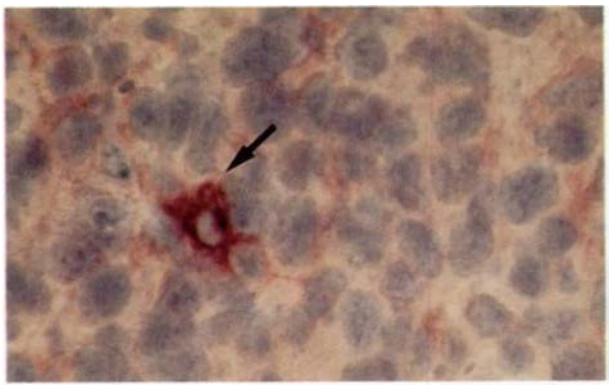


Fig. 2. Dendritic morphology of a OM1-positive cell (arrow) in the paracortical area of a lymph node. The MAb stains a membrane component. [×500; APAAP].

RESULTS

Production of Monoclonal Antibodies

Two hundred and seven hybridomas were obtained after two fusions, and 112 clones (54%) were positive in the cell-ELISA technique using microplates coated with alveolar macrophages. Following subsequent characterization of these positive supernatants by testing their reactivity (1) on microplates coated with lymphocytes or granulocytes and (2) on cytospots of alveolar macrophages, lymphocytes, or granulocytes, three MAbs were retained because of their selective specificity for alveolar macrophages. They were named respectively OM1, OM2, and OM3. These MAbs were found to be mouse IgG1 antibodies as determined by hemagglutination.

Distribution of OM1-, OM2-, and OM3-Positive Cells in Normal Tissues

Using different cell suspensions on cytospots, the MAb OM1 was found to react with all alveolar macrophages (Fig. 1) and monocytes, but not with lymphocytes as determined by im-

TABLE 1. Distribution of OM1-Positive Cells in Sheep Normal (Lymphoid and Nonlymphoid Organs) and Abnormal (Pyogranuloma) Tissue

Tissue	Position and type of positive cells
Lymph node (n = 8)[a]	Subcapsular sinus; scarce positive cells in follicles; paracortical area, medulla
Spleen (n = 2)	Red pulp, marginal zone macrophages; periarteriolar lymphocyte sheath; scarce positive cells in follicles
Thymus (n = 2)	Medulla; scarce positive cells in cortical area
Peyer's patches (n = 2)	Dome; interfollicular area
Villi (n = 4)	Lamina propria
Liver (n = 2)	Kupffer cells
Lungs (n = 2)	Alveolar macrophages; tissue macrophages
Skin (n = 3)	Histiocytes in dermis
Pyogranuloma (n = 9)	Palisade of macrophages and epithelioid cells around the necrotic center
Cell suspensions (n = 7)	Aveolar macrophages; blood: monocytes, eosinophils

[a]Number of samples examined.

munohistochemistry. This MAb reacted weakly with eosinophils as suggested by indirect immunofluorescence. The staining of OM1 on monocytes was not as strong as on alveolar macrophages. The distribution of antigen recognized by OM1 showed membrane staining, and this was confirmed by indirect immunofluorescence on live, unfixed alveolar macrophages.

In lymphoid tissues, MAb OM1 reacted with resident macrophages. This finding was based on the morphology (Fig. 2) and the localization of positive cells in the various compartments of the lymphoid organs (Table 1) and on the comparison of immunohistochemical staining with OM1 and the demonstration of acid phosphatase activity. Thus, OM1-positive cells and acid phosphatase-positive cells had comparable distributions on serial sections in lymph nodes and in spleen (Fig. 3), except for two features: (1) in lymph nodes, the number of OM1-positive cells in the cortical area was greater than the number of acid phosphatase-positive cells, and (2) the large tingible body macrophages present in follicles and strongly positive with the acid phosphatase staining were negative with MAb OM1.

In thymus, the OM1-positive cells were distributed predominantly in medulla (Fig. 4).

In nonlymphoid organs, MAb OM1 reacted with cells known to be members of the mononuclear phagocyte system

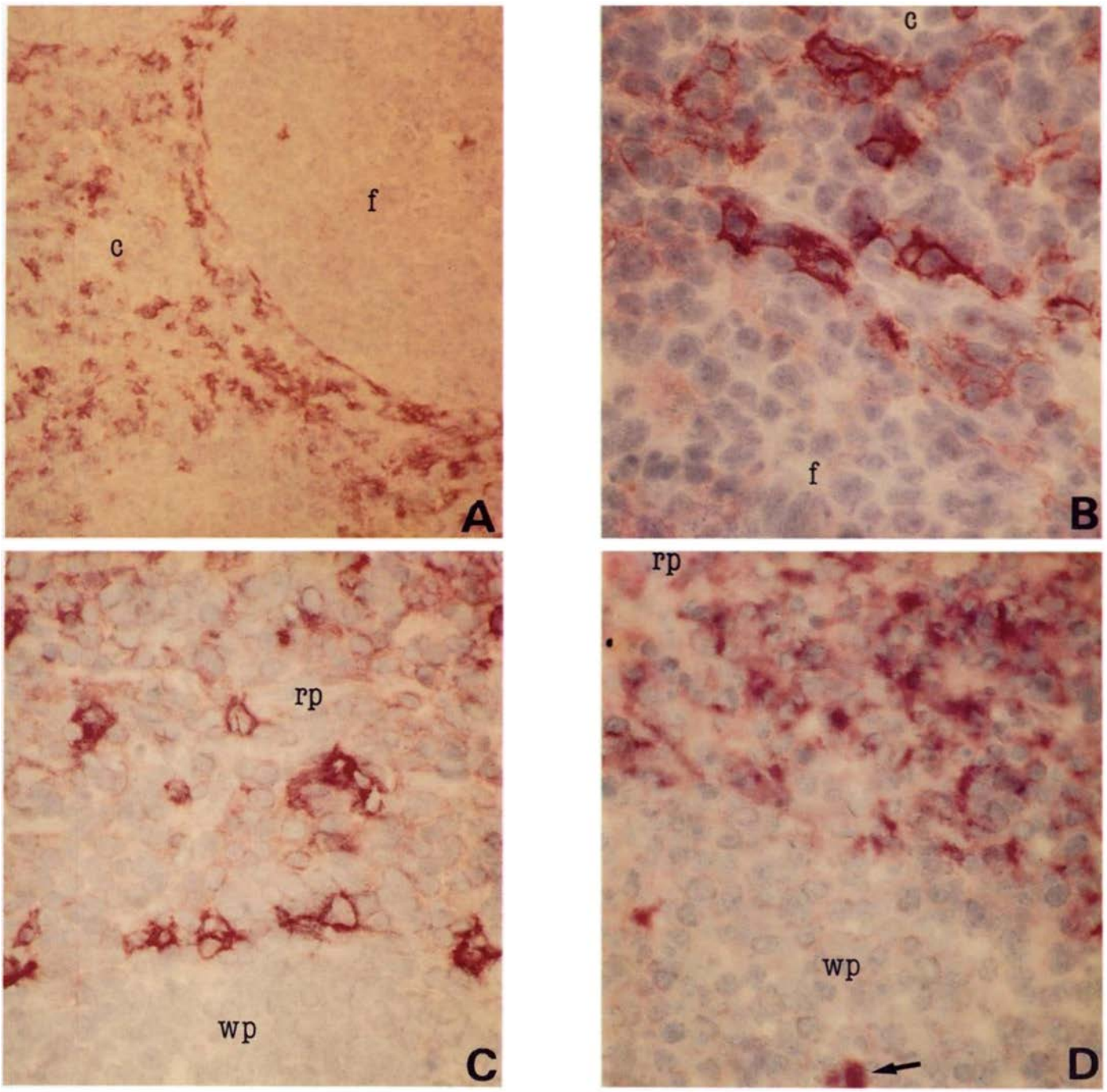


Fig. 3. Tissue distribution of MAb OM1⁺ cells stained by an APAAP technique on sections of lymph nodes and spleen. (A; ×100) Lymph node stained with OM1; (B; ×400) detail. Comparison of tissue distribution of MAb OM1+ cells stained by a APAAP technique and cells containing acid phosphatase on serial sections of spleen: (C; ×320) Spleen section stained with OM1; and (D; ×320) spleen section stained for acid phosphatase activity. f: follicle; c: cortex; wp: white pulp; rp: red pulp. Note the presence of acid phosphatase-positive cells in white pulp [arrow].

(Table 1). In the liver, Kupffer cells were positive (Fig. 5a). In the lung, all alveolar macrophages and tissue macrophages stained positive with OM1 (Fig. 5b). In the skin, dermal histiocytes were positive but epidermal Langerhans cells were not stained by MAb OM1.

On cell suspensions, MAbs OM2 and OM3 reacted with nearly all alveolar macrophages but did not stain lymphocytes, monocytes, or PMNs. In lymphoid or nonlymphoid tissues, the distribution of these two MAbs was different from that of MAb OM1. MAb OM2 labeled vascular endothelial cells in all tissues examined (Fig. 6a). MAb OM3 did not stain any cell in most of the tissues examined except in the liver, where the hepatocytes strongly reacted with this MAb (Fig. 6b).

Distribution of Macrophages in Experimentally Induced Pyogranulomas in Lambs

Using the three MAbs described above, we checked the presence of mononuclear phagocytes in the pyogranulomas induced by *C. pseudotuberculosis* in lambs. Serial sections of parotid or lateral retropharyngeal lymph nodes sampled on days 6, 12, and 28 postinoculation (PI) from nine infected lambs (three lambs/day) were examined.

On day 6 PI, numerous OM1-positive cells were detected in the pyogranulomatous lesions. These cells were strongly stained and were distributed around the necrotic center in the inner layer (Fig. 7). The OM1-positive cells were scattered in the second layer among a predominant lymphoid population. The cells positive for MAbs OM2 and OM3 were scarce at this stage of development of pyogranuloma. MAb SBU-II, which reacted with ovine MHC class II antigens, stained only a few cells in close contact with the necrotic center, whereas the numerous cells expressing class II antigens in the second cellular layer were mainly lymphocytes.

On day 12 PI, the OM1-positive cells were always mainly distributed around the necrotic center, but some positive cells also infiltrated the lymphoid cell layer (Fig. 8a). The distribution of OM1-positive cells was comparable to that observed with the acid phosphatase activity (Fig. 8b). The staining by MAb SBU-II showed that most macrophages in the pyogranuloma expressed class II antigens, as did numerous lymphocytes. When the lesions present in the three lambs necropsied on day 12 were analyzed, the other two MAbs enabled two types of pyogranulomas to be distinguished. The first was characterized by the presence of numerous macrophages strongly stained by MAb OM2 and distributed around the necrotic center, whereas the very scarce $OM3^+$ cells were disseminated throughout the wall of the pyogranuloma. In contrast, the second type was characterized by the presence of numerous $OM3^+$ cells disseminated throughout the wall of the pyogranuloma, whereas $OM2^+$ cells were scarce.

On day 28 PI, a palisade of macrophages and epithelioid cells in close contact with the necrotic center was very strongly stained by MAb OM1 (Figs. 9a and 10a). The pattern of staining by MAb SBU-II was slightly different from that observed on days 6 and 12 PI because all cells constitut-

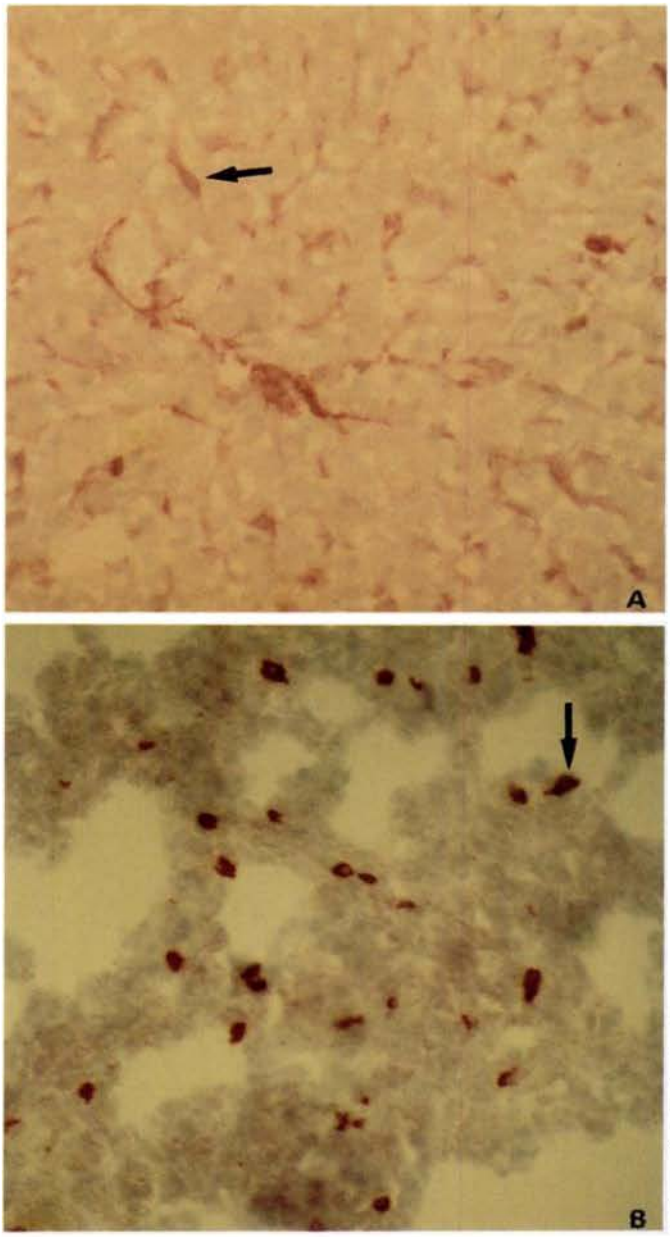


Fig. 5. Distribution of OM1-positive cells in the liver and the lung. (A) Liver; the Kuppfer cells (arrow) are positive with OM1; (B) lung; the alveolar macrophages (arrow) are strongly stained with OM1 [×80; APAAP].

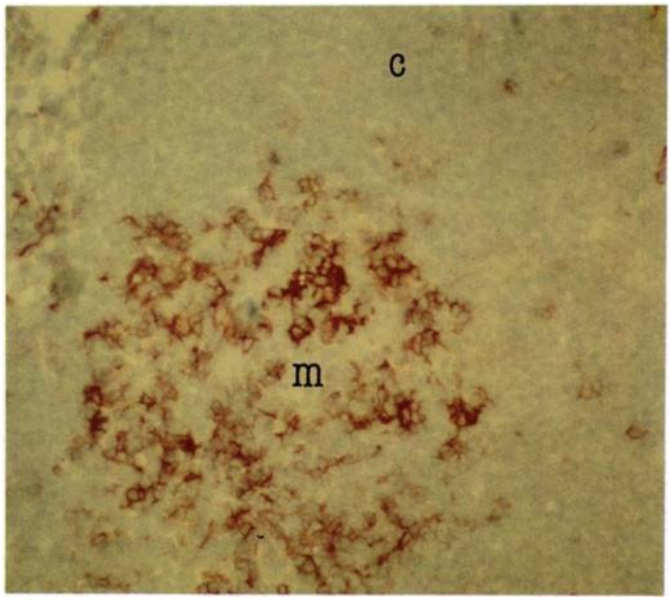
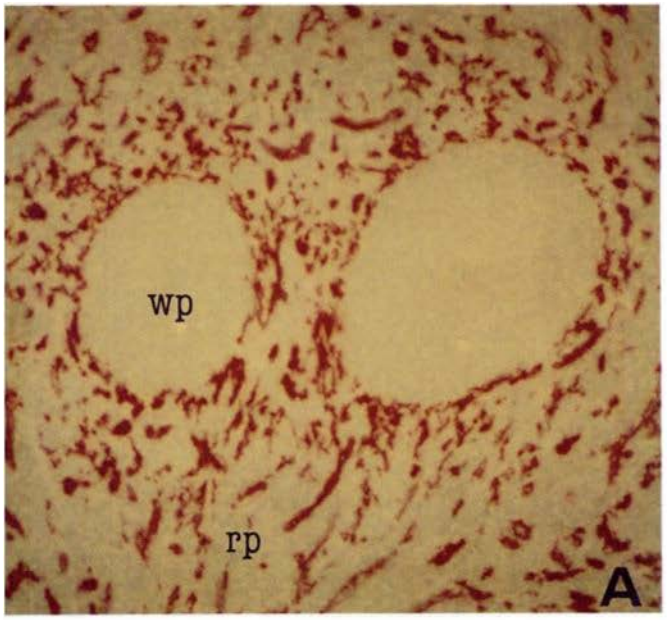
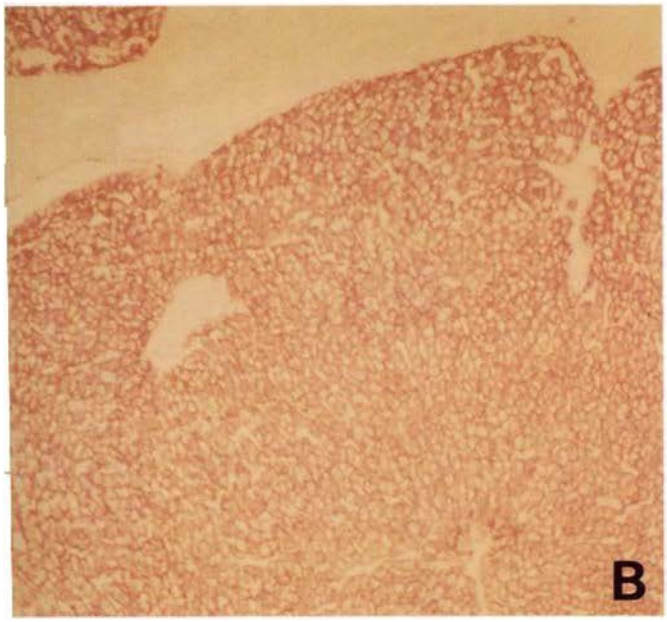


Fig. 4. Distribution of OM1-positive cells in thymus [×100; APAAP]. c: cortex; m: medulla.





ig. 6. Immunohistochemical staining with MAbs OM2 (A) and OM3 (B). ۱) Spleen section; endothelial structures are stained with OM2; wp: white ılp; rp: red pulp [×50; APAAP]; (B) liver section; hepatocytes are stained ith OM3. [×40; APAAP].

ıg the macrophage palisade were positive, as were most cells ı the lymphoid layer (Figs. 9b and 10b). The two histologi-ıl patterns observed for MAbs OM2 and OM3 (Figs. 9 and)) were identical to those observed on day 12 PI, with two ıain features: (1) the epithelioid cells, easily recognizable ecause of their typical morphology, were strongly stained by IAb OM2, whereas they were negative with OM3 (Figs. 9c ıd 9d); and (2) the typical distribution of macrophage sub-:ts stained by OM2 and OM3 was that OM2-positive cells ere distributed only in close contact with the necrotic :nter (Figs. 9c and 10c), whereas OM3-positive cells were stributed throughout the pyogranuloma (Fig. 10d).

The surface phenotype of the cells in the centers of pyo-·anulomas could not be evaluated because these necrotic enters stained nonspecifically with most of the antibodies sed or were lost during the immunohistochemical procedure.

DISCUSSION

Distribution of the MAbs in Normal Tissues

We have described the obtention and cell binding of three MAbs raised against ovine macrophages. The tissue distribution of MAbs, OM1, OM2, and OM3 was determined in various sheep organs before testing their reactivity with inflammatory macrophages recruited in experimentally induced pyogranulomatous lesions of lambs inoculated with *C. pseudotuberculosis*.

MAb OM1 recognizes a membrane antigen expressed by most macrophages of the sheep, as determined by tissue distribution, morphology of the cells, and comparison with the acid phosphatase staining in lymphoid organs. MAb OM1 staining intensity increases with the differentiation stage of the monocyte/macrophage lineage, as already described with other MAbs such as MOMA-2 in mouse [32] and BRL-M1, CM-1, and mAb-24 in humans [23, 24]. The possibility that this MAb recognizes other hematopoietic cells in tissues cannot be excluded because of its observed reactivity with eosinophils in cell suspensions of peripheral blood leukocytes (Table 1). Among the resident macrophage subpopulations tested, a few did not react with OM1, such as the cortical macrophages in the thymus or tingible body macrophages in follicles of lymphoid organs. This differential expression of macrophages markers according to their tissue location has been described in thymus [10], spleen [15], lymph nodes [8, 15], and nonlymphoid organs of different species [3, 46]. Other macrophage populations were not tested in this study, in particular the microglial cells of the brain and the resident bone marrow macrophages. Except for the foregoing reservations, MAb OM1 resembles ED1 in the rat [15], F4/80 in mouse [26, 34], and CM-1 and RFD2 in humans [23, 42] and can be proposed as a panmacrophage marker in sheep.

The tissue distribution of the two other antibodies showed that (1) they recognized a subpopulation of alveolar macrophages and (2) they were not specific for the monocyte/macrophage lineage. Thus, MAb OM2 appeared to label vascular endothelium; this antigenic similarity between the vascular endothelium and subsets of macrophages has already been

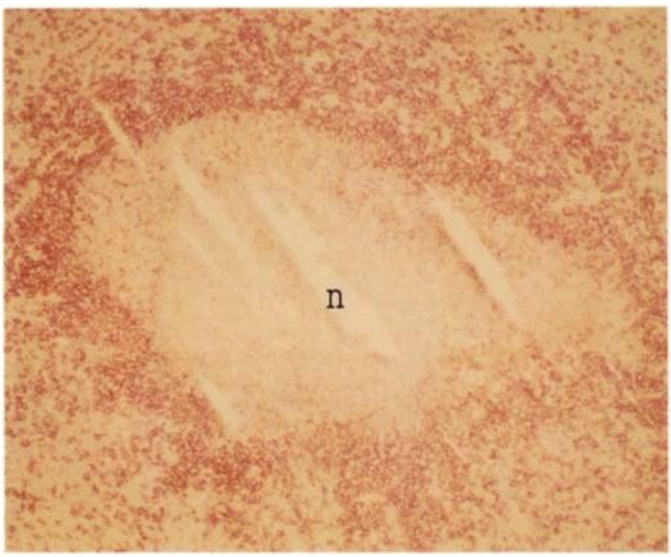


Fig. 7. Immunohistochemical staining with MAb OM1 on a lymph node with a 6-day-old pyogranuloma. Note the distribution of OM1-positive macrophages which are predominantly distributed in a mantle surrounding the necrotic center (n) [×32; APAAP].

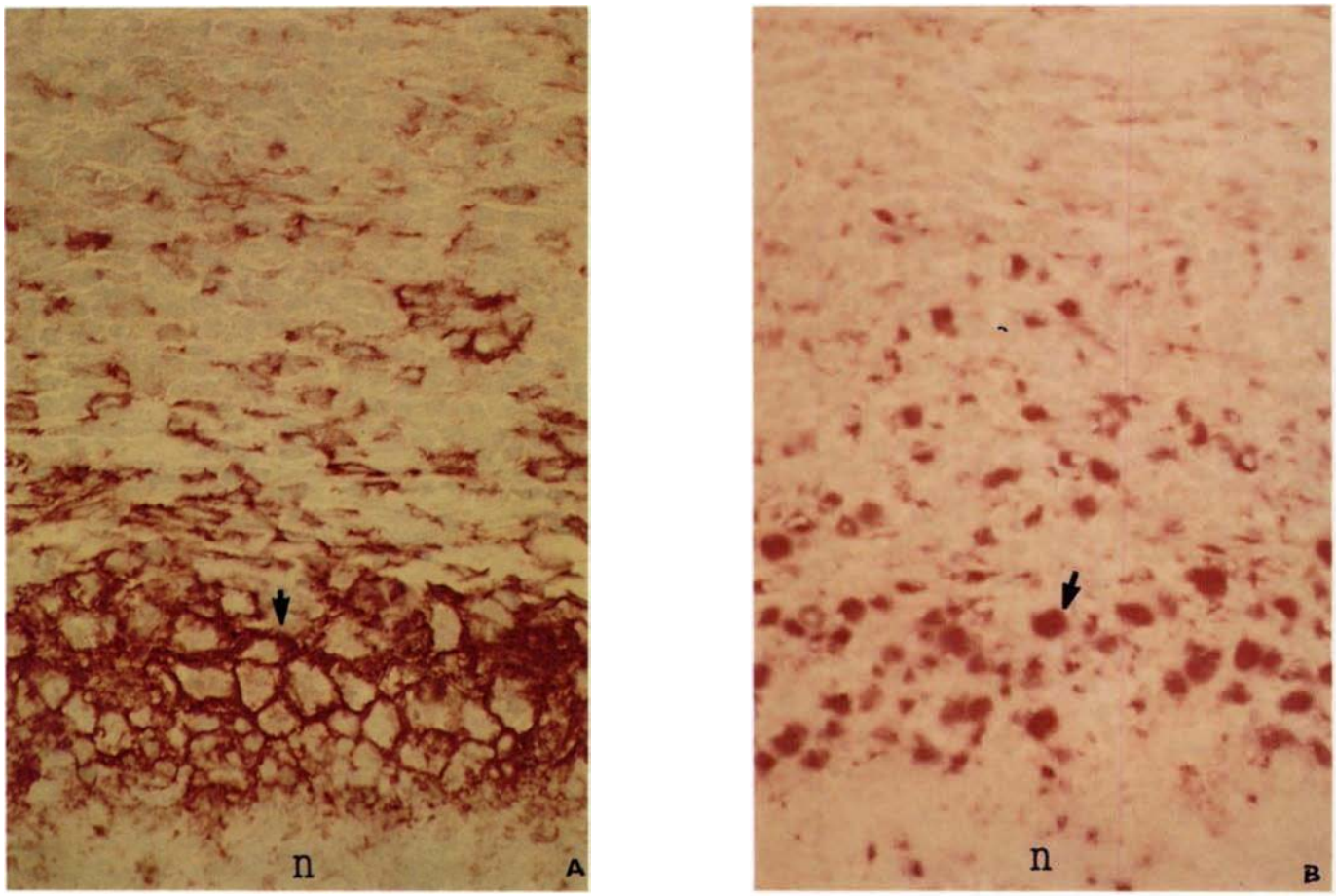


Fig. 8. Distribution of OM1-positive macrophages (arrow) and positive cells for acid phosphatase (arrow) in a lymph node with a 28-day-old pyogranuloma. (A; APAAP) Pyogranuloma stained with OM1; (B) serial section stained for acid phosphatase activity. n: necrotic center [×160].

described in humans with MAbs OKM5, UCHM1, and SMϕ [25, 30]; in rat with MAbs R2-2B1 and MRC OX-43 [27, 45]; and in mice [29]. Moreover, it has been shown that endothelial cells and macrophages have some functional similarities despite their separate embryologic origins. More surprising was the staining of hepatocytes by MAb OM3; to our knowledge, there has been no previous report of such cross-reactivity between a subset of macrophages and hepatocytes.

Thus, two conclusions can be drawn from these results: (1) the three MAbs do no label the dendritic cells, suggesting that either the Langerhans-dendritic cell lineage is distinct from the mononuclear phagocyte system in sheep, as already suggested in other species [4, 31, 44, 56], or that the capacity of these cells to bind the MAbs is lost in the process of differentiation; and (2) there is great heterogeneity in the ovine mononuclear phagocyte system, which made it possible to use these MAbs to analyze the cellular composition of pyogranuloma.

The Mononuclear Phagocytes in Pyogranulomatous Inflammation

From the immunohistochemical examination of infectious pyogranuloma induced in lambs, the major finding that emerges is that cells of the mononuclear phagocyte system, recognized by MAb OM1, massively infiltrate the lesion from day 6 to day 28 PI. The importance of macrophages surrounding a suppurative center in such lesions agrees with results of earlier studies [14, 16, 19, 33, 52] and emphasizes the denominations of pyogranuloma or suppurative granuloma used to designate these lesions [21, 38]. Obviously, this does not exclude the participation of other cells such as eosinophils stained by MAb OM1, which have been described in granulomas induced by parasites [22], or dendritic cells [41, 55].

Following the recruitment of monocyte-derived cells to the inflammatory sites, two progressions could be observed leading to two types of pyogranulomas. In the first, a large proportion of epithelioid cells [36, 49, 50, 53] stained by MAbs OM1 and OM2 could be observed early, from day 6 PI, and increased in size and number until day 28. In this case, the $OM3^+$ cells were scarce. The second was characterized by a low proportion of epithelioid cells, whereas numerous $OM3^+$ macrophages could be observed throughout the pyogranuloma. These results suggest that these different histological patterns in *C. pseudotuberculosis*-induced pyogranulomas may depend on the immunological status of the host. This histological spectrum could be compared with that described in mycobacterial diseases [12, 47, 48, 50].

Another picture that emerges from this study is that macrophages involved in the pyogranuloma express high levels of MHC class II antigens in the late phases of development of the lesions as observed on day 28 PI. Although the presence

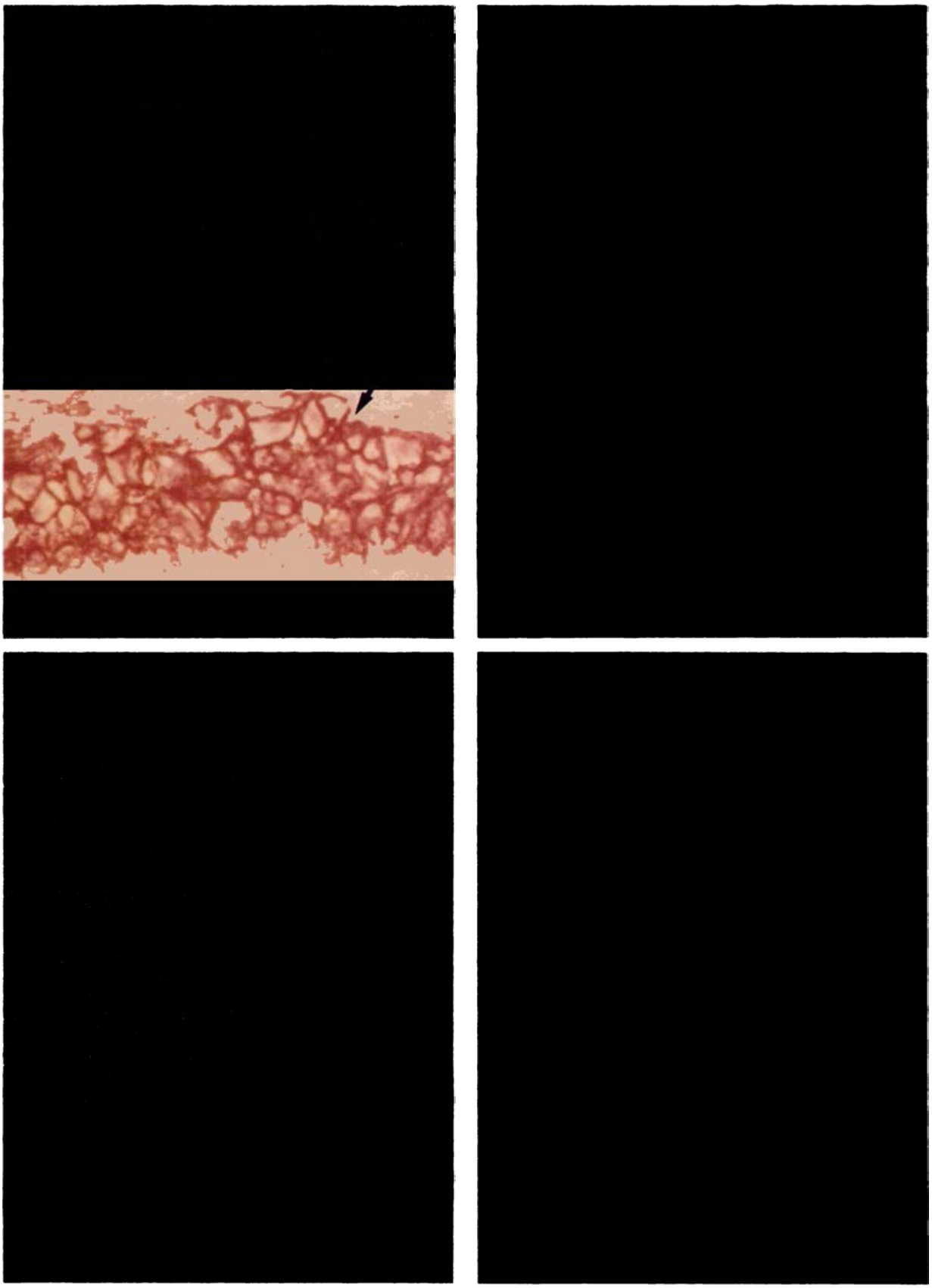


Fig. 9. Macrophage subsets and MHC class II-positive cells on serial sections of a lymph node with a 28-day-old pyogranuloma. (A) Staining with MAb OM1. All the macrophages distributed in a palisade around the necrotic center (n) are positive; (B) Staining with MAb SBU-II raised against the class II antigens. Macrophages and numerous lymphocytes are positive; (C) Staining with MAb OM2. The numerous positive cells are distributed in close contact with the necrotic center and have an epithelioid cell morphology; (D) Staining with MAb OM3. The OM3-positive (arrow) cells are very scarce in comparison with the OM2-positive cells. L: cellular layer predominantly composed of lymphocytes [×160; APAAP].

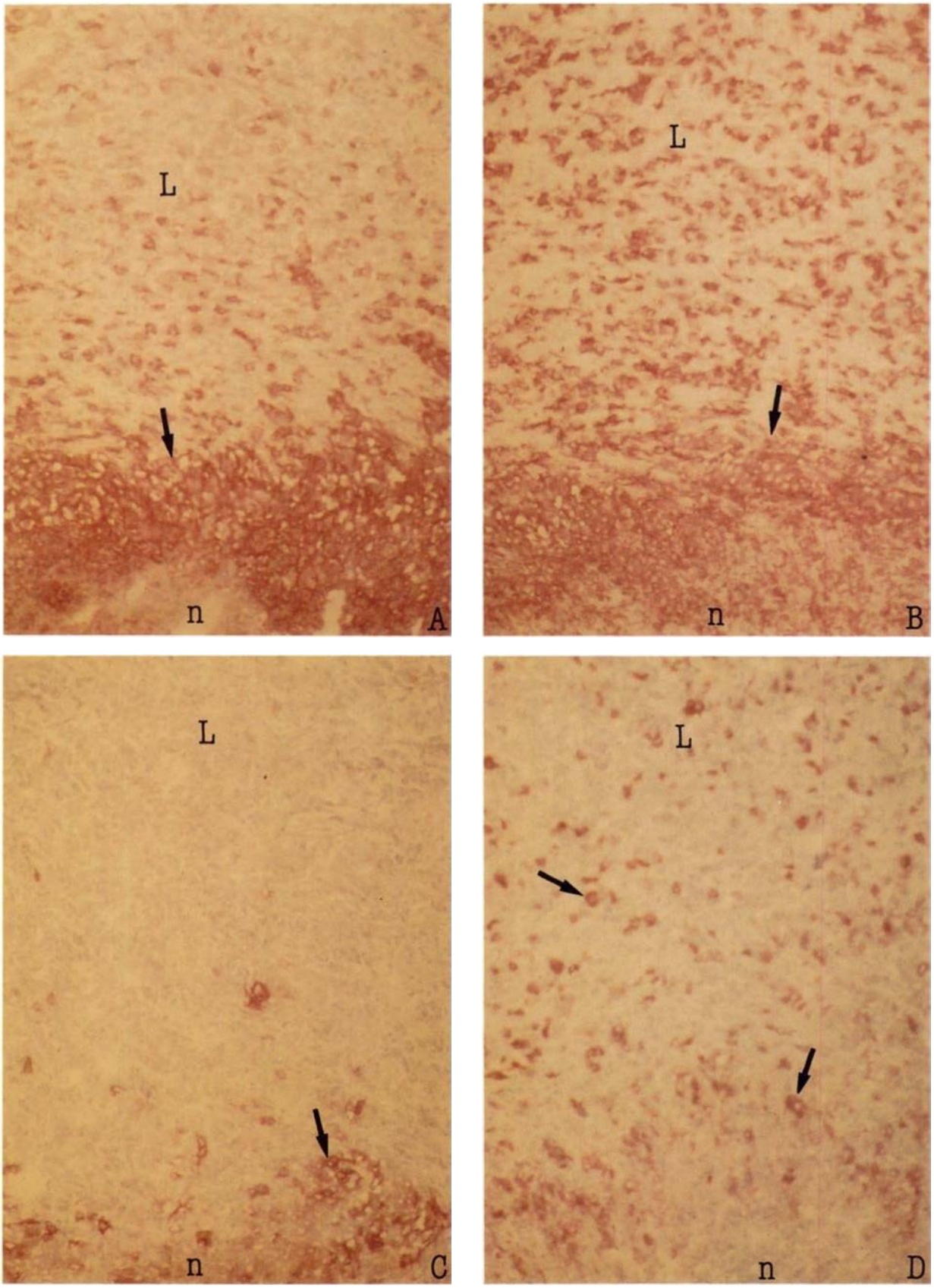


Fig. 10. Macrophage subsets and MHC class II-positive cells on serial sections of a lymph node with a 28-day-old pyogranuloma. (A) Staining with MAb OM1. All the macrophages distributed in a palisade around the necrotic center (n) are positive; (B) Staining with MAb SBU-II raised against the class II antigens. Macrophages and numerous lymphocytes are positive; (C) Staining with MAb OM2. The scarce positive cells are distributed in close contact with the necrotic center and have an epithelioid cell morphology (arrow); (D) Staining with MAb OM3. The numerous OM3 positive (arrow) cells are distributed throughout the pyogranuloma. L: cellular layer predominantly composed of lymphocytes [×80; APAAP].

of these immunoregulatory molecules is not a marker for "activation" per se, the intensity and extent of class II antigen expression, as described in naturally occurring *C. pseudotuberculosis* lesions [16] or in hepatic granulomas [2], illustrate the participation of monocyte-derived cells in accessory cell functions [51].

Although the nature of the antigens recognized by MAbs OM1, OM2, and OM3 and their roles in functional aspects of cells of the ovine mononuclear phagocyte system need further study, the present results show that these MAbs are very useful for examining the heterogeneity and the involvement of macrophages in granulomatous lesions.

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REFERENCES

1. Adams, D.O., and Hamilton, T.A. Phagocytic cells: cytotoxic activities of macrophages. In *Inflammation: Basic Principles and Clinical Correlates* (Gallin, J.I., Goldstein, I.M., and Snyderman, R., Eds.). New York: Raven Press, p. 471, 1988.
2. Allen, J.B., and Wilder, R.L. Variable severity and Ia antigen expression in streptococcal-cell-wall-induced hepatic granulomas in rats. *J. Immunol.* **55,** 674, 1987.
3. Andreesen, R., Gadd, S., Costabel, U., Leser, H.G., Speth, V., Cesnik, B., and Atkins, R.C. Human macrophage maturation and heterogeneity: restricted expression of late differentiation antigens in situ. *Cell Tissue Res.* **253,** 271, 1988.
4. Austyn, J.M. Lymphoid dendritic cells. *Immunology* **62,** 161, 1987.
5. Baumgarten, H. A cell ELISA for the quantitation of leukocyte antigens. Requirements for calibration. *J. Immunol. Methods* **94,** 91, 1986.
6. Brown, C.C., and Olander, H.J. Caseous lymphadenitis of goats and sheep: a review. *Vet. Bull.* **57,** 1, 1987.
7. Carlson, G.P., and Kaneko, J.J. Isolation of leukocytes from bovine peripheral blood. *Proc. Soc. Exp. Biol. Med.* **142,** 853, 1973.
8. Chao, D., and MacPherson, G.G. Lymph node macrophage heterogeneity: the phenotypic and functional characterization of two distinct populations of macrophages from rat lymph node. *Eur. J. Immunol.* **19,** 1273, 1989.
9. Charley, B., Frénove, B., and Villiers, P. Description et efficacité d'une méthode modifiée de lavage pulmonaire chez le porc anesthésié. *Ann. Rech. Vet.* **11,** 209, 1980.
10. Colic, M., Popovic, L.J., Gasic, S., Dragojevic-Simic, V., Milicevic, N.M., and Matanovic, D. Immunohistochemical characterization of rat thymic non-lymphoid cells. II. Macrophages and granulocytes defined by monoclonal antibodies. *Immunology* **69,** 416, 1990.
11. Cordell, J.L., Falini, B., Erber, W.N., Ghosh, A.K., Abdulaziz, Z., Macdonald, S., Pulford, K.A.F., Stein, H., and Mason, D.Y. Immunoenzymatic labeling of monoclonal antibodies using immune complexes of alkaline phosphatase and monoclonal anti-alkaline phosphatase (APAAP complexes). *J. Histochem. Cytochem.* **32,** 219, 1984.
12. Dannenberg, A.M. Immune mechanisms in the pathogenesis of pulmonary tuberculosis. *Rev. Infect. Dis.* **11** (S2), 369, 1989.
13. Dannenberg, A.M., and Suga, M. Histochemical stains for macrophages in cell smears and tissue sections: β-galactosidase, acid phosphatase, nonspecific esterase, succinic dehydrogenase, and cytochrome oxidase. In *Methods for Studying Mononuclear Phagocytes* (Adams, D.O., Edelson, P.J., and Koren, H.S., Eds.). New York: Academic Press, p. 375, 1981.
14. Dannenberg, A.M., Meyer, O.T., Esterly, J.R., and Kambara, T. The local nature of immunity in tuberculosis illustrated histochemically in dermal BCG lesions. *J. Immunol.* **100,** 931, 1968.
15. Dijkstra, C.D., Döpp, E.A., Joling, P., and Kraal, G. The heterogeneity of mononuclear phagocytes in lymphoid organs: distinct macrophage subpopulations in the rat recognized by monoclonal antibodies ED1, ED2 and ED3. *Immunology* **54,** 589, 1985.
16. Ellis, J.A. Immunophenotype of pulmonary cellular infiltrates in sheep with visceral caseous lymphadenitis. *Vet. Pathol.* **25,** 362, 1988.
17. Garrett, K.C., Richerson, H.B., and Hunninghake, G.W. Pathogenesis of the granulomatous lung diseases. II. Mechanisms of granuloma formation. *Am. Rev. Respir. Dis.* **130,** 477, 1984.
18. Goding, J.W. *Monoclonal antibodies: Principles and Practice,* 2nd ed. London: Academic Press, p. 315, 1986.
19. Goldberger, A.C., Lipsky, B.A., and Plorde, J.J. Suppurative granulomatous lymphadenitis caused by *Corynebacterium ovis (pseudotuberculosis). Am. J. Clin. Pathol.* **76,** 486, 1981.
20. Gordon, S. Biology of the macrophage. *J. Cell Sci. [Suppl.]* **4,** 267, 1986.
21. Guilloteau, L., Pépin, M., Pardon, P., and LePape, A. Recruitment of 99m-technetium- or 111-indium-labelled polymorphonuclear leucocytes in experimentally induced pyogranulomas in lambs. *J. Leuk. Biol.* **48,** 343, 1990.
22. Hahn, H., and Kaufmann, S.H.E. The role of cell-mediated immunity in bacterial infections. *Rev. Infect. Dis.* **3,** 1221, 1981.
23. Hofman, F.M., Lopez, D., Husmann, L., Meyer, P.R., and Taylor, C.R. Heterogeneity of macrophage populations in human lymphoid tissue and peripheral blood. *Cell. Immunol.* **88,** 61, 1984.
24. Hogg, N., and Selvendran, Y. An anti-human monocyte/macrophage monoclonal antibody, reacting most strongly with macrophages in lymphoid tissue. *Cell. Immunol.* **92,** 247, 1985.
25. Hogg, N., MacDonald, S., Slusarenko, M., and Beverley, P.C.L. Monoclonal antibodies specific for human monocytes, granulocytes and endothelium. *Immunology* **53,** 753, 1984.
26. Hume, D.A., Robinson, A.P., MacPherson, G.G., and Gordon, S. The mononuclear phagocyte system of the mouse defined by immunohistochemical localization of antigen F4/80. Relationship between macrophages, Langerhans cells, reticular cells, and dendritic cells in lymphoid and hematopoietic organs. *J. Exp. Med.* **158,** 1522, 1983.
27. Ishii, Y., Matsuura, A., Iwaki, H., Takami, T., and Kikuchi, K. Two closely related antigens expressed on granulocytes, macrophages and some reticular elements in rat lymphoid tissues: characterization by monoclonal antibodies. *Immunology* **51,** 477, 1984.
28. Johnson, G.D., and Holborow, E.J. Preparation and use of fluorochrome conjugates. In *Handbook of Experimental Immunology.* Vol. 1. *Immunochemistry.* 4th ed. (Weir, D.M., Herzenberg, L.A., Blackwell, C., and Herzenberg, L.A., Eds.). Oxford: Blackwell Scientific Publications, p. 28-1, 1986.
29. Jutila, M.A., Kroese, F.G., Jutila, K.L., Stall, A.M., Fiering, S., Herzenberg, L.A., Berg, E.L., and Butcher, E.C. Ly-6C is a monocyte/macrophage and endothelial cell differentiation antigen regulated by interferon-gamma. *Eur. J. Immunol.* **18,** 1819, 1988.
30. Knowles, D.M., Tolidjian, B., Marboe, C., D'Agati, V., Grimes, M., and Chess, L. Monoclonal anti-human monocyte antibodies OKM1 and OKM5 possess distinctive distributions including differential reactivity with vascular endothelium. *J. Immunol.* **132,** 2170, 1984.
31. Kraal, G., Breel, M., Janse, M., and Bruin, G. Langerhans' cells, veiled cells, and interdigitating cells in the mouse recognized by a monoclonal antibody. *J. Exp. Med.* **163,** 981, 1986.
32. Kraal, G., Rep, M., and Janse, M. Macrophages in T and B cell compartments and other tissue macrophages recognized by monoclonal antibody MOMA-2. An immunohistochemical study. *Scand. J. Immunol.* **26,** 653, 1987.
33. Mathew, R.C., Katayama, I., Gupta, S.K., Curtis, J., and Turk, J.L. Analysis of cells of the mononuclear phagocyte series in ex-

perimental mycobacterial granulomas by monoclonal antibodies. *Infect. Immun.* **39,** 344, 1983.
34. McGarry, M., Stewart, S.J., and Stewart, C.C. Murine eosinophils are F4/80 positive. *J. Leuk. Biol. [Suppl.]* **1,** 17 (abstract 9), 1990.
35. Miyasaka, M., and Trnka, Z. Sheep as an experimental model for immunology: immunological techniques in vitro and in vivo. In *Immunological Methods* (Lefkovits, I., and Pernis, B., Eds.). London: Academic Press, vol. 3, p. 403, 1985.
36. Munro, C.S., Campbell, D.A., Collings, L.A., and Poulter, L.W. Monoclonal antibodies distinguish macrophages and epithelioid cells in sarcoidosis and leprosy. *Clin. Exp. Immunol.* **68,** 282, 1987.
37. Pépin, M., Boisramé, A., and Marly, J. *Corynebacterium pseudotuberculosis*: biochemical properties, production of toxin and virulence of ovine and caprine strains. *Ann. Rech. Vét.* **20,** 111, 1989.
38. Pépin, M., Fontaine, J.J., Pardon, P., Marly, J., and Parodi, A.L. Histopathology of the early phase during experimental *Corynebacterium pseudotuberculosis* infection in lambs. *Vet. Microbiol.* **29,** 123, 1991
39. Pépin, M. Pardon, P., Lantier, F., Marly, J., Levieux, D., and Lamand, M. Experimental *Corynebacterium pseudotuberculosis* infection in lambs: kinetics of bacterial dissemination and inflammation. *Vet. Microbiol.* **26,** 381, 1991.
40. Pépin, M., Pardon, P., Marly, J., and Lantier, F. *Corynebacterium pseudotuberculosis* infection in adult ewes by inoculation in the external ear. *Am. J. Vet. Res.* **49,** 459, 1988.
41. Poulter, L.W., and Janossy, G. The involvement of dendritic cells in chronic inflammatory disease. *Scand. J. Immunol.* **21,** 401, 1985.
42. Poulter, L.W., Campbell, D.A., Munro, C., and Janossy, G. Discrimination of human macrophages and dendritic cells by means of monoclonal antibodies. *Scand. J. Immunol.* **24,** 351, 1986.
43. Puri, N.K., Mackay, C.R., and Brandon, M.R. Sheep lymphocyte antigens (OLA). II. Major histocompatibility complex class II molecules. *Immunology* **56,** 725, 1985.
44. Radzun, H.J., Kreipe, H., Zavazava, N., Hansmann, M.-L., and Parwaresh, M.R. Diversity of the human monocyte/macrophage system as detected by monoclonal antibodies. *J. Leuk. Biol.* **43,** 41, 1988.
45. Robinson, A.P., White, T.M., and Mason, D.W. MRC OX-43: a monoclonal antibody which reacts with all vascular endothelium in the rat except that of brain capillaries. *Immunology* **57,** 231, 1986.
46. Rumpold, H., Förster, G., Swetly, P., and Riedl, M. Antigenic heterogeneity of rat macrophages. A monoclonal antibody reacting only with alveolar but not with other types of macrophages. *Immunology* **45,** 637, 1982.
47. Smith, D.W., and Wiegeshaus, E.H. What animal models can teach us about the pathogenesis of tuberculosis in humans. *Rev. Infect. Dis.* **11**(S2), 385, 1989.
48. Spector, W.G. Experimental granulomas. *Pathol. Res. Pract.* **175,** 110, 1982.
49. Sutton, J.S., and Weiss, L. Transformation of monocytes in tissue culture into macrophages, epithelioid cells, and multinucleated giant cells. An electron microscope study. *J. Cell Biol.* **28,** 303, 1966.
50. Turk, J.L. Current status review: a comparison of secretory epithelioid cells and phagocytosing macrophages in experimental mycobacterial granulomas. *Br. J. Exp. Pathol.* **70,** 589, 1989.
51. Unanue, E.R. Antigen-presenting function of the macrophage. *Annu. Rev. Immunol.* **2,** 395, 1984.
52. Van den Oord, J.J., De Wolf-Peeters, C., and Desmet, V.J. Cellular composition of suppurative granulomas: an immunohistochemical analysis of suppurative granulomatous lymphadenitis. *Hum. Pathol.* **16,** 1009, 1985.
53. Van der Rhee, H.J., Van der Burgh-de Winter, C.P.M., and Daems, W.T. The differentiation of monocytes into macrophages, epithelioid cells, and multinucleated giant cells in subcutaneous granulomas. *Cell Tissue Res.* **197,** 355, 1979.
54. Van Furth, R., and Cohn, Z.A. The origin and kinetics of mononuclear phagocytes. *J. Exp. Med.* **128,** 415, 1968.
55. Verghese, S., Healey, D.G., Curtis, J., and Turk, J. L. Accessory cell function of dendritic cells from lymph nodes containing *Mycobacterium leprae* induced granulomas. *Int. Arch. Allergy Appl. Immunol.* **87,** 392, 1988.
56. Wacker, H., Radzun, H.J., and Parwaresch, M.R. KI-M2R, a new specific monoclonal antibody, discriminates tissue macrophages from reticulum cells and monocytes in vivo and in vitro. *J. Leuk. Biol.* **38,** 509, 1985.
57. Williams, G.T., and Williams, W.J. Granulomatous inflammation. A review. *J. Clin. Pathol.* **36,** 723, 1983.