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Identification of an inducible surface molecule specific to fusing macrophages

(giant cells/osteoclasts)

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ABSTRACT **Multinucleated giant cells and osteoclasts arise through the fusion of mononuclear phagocyte precursors. To elucidate the mechanism by which cells of monocytic lineage fuse and differentiate into giant cells and osteoclasts, we hypothesized that, as with other cell fusion events, specific surface molecules mediate the adhesion/fusion process. It has been observed that macrophages can be induced to fuse with one another in response to specific stimuli or when placed in a specific microenvironment. The formation of giant cells is primarily associated with chronic inflammatory reactions and tumors, while osteoclasts differentiate on bone which they resorb. The fact that, under normal conditions, macrophages and monocytes fail to fuse in regions and tissues where they are present in large numbers suggests the regulated and transient expression of potential fusion molecules. To identify such a fusion-associated molecule, we established a macrophage fusion assay and generated monoclonal antibodies (mAbs) that alter the fusion of macrophages *in vitro*. We selected four mAbs that each had the ability to block the fusion but not the aggregation of macrophages *in vitro*. All four antibodies recognize surface proteins of 150 kDa. The expression of the antigens recognized by all four mAbs is restricted to macrophages that have been induced to fuse *in vitro* and *in vivo* and is inducible, transient, and regulated, as neither nonfusing macrophages nor macrophages fused *in vitro* express these antigens. These results support the hypothesis that macrophage fusion is mediated by specific fusion/adhesion molecules and also provide a means to study the molecular mechanisms of macrophage fusion.**

Mononuclear phagocytes have the potential, in specific instances, to fuse and differentiate into either osteoclasts or multinucleated giant cells. These cells are primarily associated with bone resorption and chronic inflammatory reactions, respectively. Although these cells are distinct, it is reasonable to assume that the fusion mechanism of their precursors, which belong to the mononuclear phagocyte lineage, is similar to that occurring in virus–cell, myoblast–myoblast, and sperm–oocyte fusion. While the molecular events involved in macrophage fusion remain enigmatic, the understanding of the mechanisms by which viruses fuse with host cells to introduce their nucleic acids has made some recent progress (1). It is now well established that the binding and fusion of viruses with host cells is mediated by viral proteins which use cell surface molecules as viral receptors. Virus and host cell plasma membrane binding is therefore mediated by a "receptor–ligand" type of interaction. For instance, gp120 from the human immunodeficiency virus (HIV), which causes AIDS, binds CD4 molecules on T lymphocytes and macrophages (2, 3). Simultaneously, gp40, which arises from the same precursor protein as gp120—i.e., gp160—is believed to trigger fusion by virtue of its stretch of hydrophobic amino acids known as the fusion peptide. To bring about fusion between two plasma membranes requires both an attachment mechanism that possibly stabilizes interacting membranes and a fusion peptide that perturbs the host cell plasma membrane. The viral molecule gp160 acts, upon cleavage, both as a ligand and a fusion molecule.

Recently, antibodies with anti-fusion activity directed against myoblasts (4) and sperm cells (5) have been generated. These antibodies recognize surface proteins that may mediate the actual adhesion/fusion process. Importantly, the antigen recognized by the anti-sperm cell antibody has been shown to contain an integrin ligand domain and a putative fusion peptide (6, 7). This is an indication that mammalian cell fusion may be mediated, like virus–cell fusion, by cell surface proteins which have the capacity to act in concert as a ligand and a fusion molecule.

Because the molecular mechanisms controlling macrophage fusion remain elusive, we set out to identify molecules which mediate macrophage fusion. We hypothesized that, similar to the mechanism of virus–cell and sperm–oocyte fusion, specific surface proteins mediate the adhesion/fusion process of macrophages. Since the fusion of macrophages is restricted to specific microenvironments and disease states, we also hypothesized that the expression of fusion-related proteins should be inducible, transient, and regulated. To identify such molecules in macrophages, we generated monoclonal antibodies (mAbs) that alter fusion *in vitro*. The present study reports the identification of a surface molecule whose expression is inducible, transient, and specific for macrophages induced to fuse.

MATERIAL AND METHODS

Cells. Rat alveolar macrophages were obtained from 12-week-old Fisher rats (Charles River Breeding Laboratories) by tracheobronchial lavage and cultured under fusogenic conditions as described (8–11). Osteoclast-like cells were elicited *in vivo* by implanting syngeneic bone particles intramuscularly for 10 days in 12-week-old Sprague–Dawley rats (Charles River Breeding Laboratories) as described (8, 9).

Chemicals. Unless otherwise stated, all chemicals were purchased from Sigma.

Antibodies. C57BL/6J mice were immunized with an equal mix of unfixed and fixed fusing alveolar macrophages (12), and their spleens were fused with myeloma cells Ag8 to generate hybridomas. All hybridomas that altered fusion displayed anti-fusion activity. To select the hybridomas which produce IgGs which recognize surface determinants, hybridoma supernatants were next tested by indirect immunofluorescence. Four mAbs (10C4, 10C5, 10B11, and 12D6) were selected. All four are the IgG1 isotype, block fusion, and detect surface

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Abbreviations: mAb, monoclonal antibody; HIV, human immunodeficiency virus; FITC, fluorescein isothiocyanate; Cy3, indocarbocyanine; $DiOC_{16}$, 1,1′-dihexadecyloxacarbocyanine; FCS, fetal calf serum.

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determinants whose expression is specific for fusing macrophages. mAb-typing kits were purchased from The Binding Site (San Diego). Goat anti-mouse IgG and fluorescein isothiocyanate (FITC)-conjugated $F(ab')_2$ goat anti-mouse IgG (heavy and light chains) were obtained from Boehringer Mannheim. Sheep anti-mouse Fab fragments conjugated with peroxidase were purchased from Biosys (Compiègne, France). Indocarbocyanine (Cy3)-conjugated $F(ab')_2$ goat anti-mouse IgG (heavy and light chains) were obtained from Jackson ImmunoResearch. Mouse IgG1 standards were obtained from Fisher Scientific. Mouse anti-rat macrophage surface antigens were obtained from Serotec (Harlan Bioproducts for Science, Indianapolis) and included the following: anti-CD4 (W3/25), anti-CD44 (MRC OX8), and anti-class II (RT1B), which are of the IgG1 isotype.

Fluorescent Labeling of Macrophage Membranes. Macrophage membranes were labeled by using a modification of the protocol published by Weston and Parish (13). Macrophages were suspended on ice at a density of 5×10^7 cells per ml in an isoosmotic concentration of mannitol in H_2O equilibrated at pH 7.4. The fluorescent dye 1,1'-dihexadecyloxacarbocyanine perchlorate ($DiOC_{16}$, Molecular Probes) was dissolved in ethanol and added to the cell suspension at a final concentration of 2 μM. The cells were incubated for 15 min at 37°C, and the reaction was stopped by placing the cells on ice. The cells were washed with MEME supplemented with 10% (vol/vol) fetal calf serum (FCS) and cultured under fusogenic conditions as described (8–11).

Immunolocalization. Tissues were prepared from both control and experimental rats implanted with bone particles (8, 9). The implants and the rat tissues were collected and processed in one of two ways as follows:

(*i*) Tissues were cut into fragments of 2–4 mm^3, fixed with 4% formaldehyde for an additional 4 h at 4°C, washed, and cryoprotected for 1 h in phosphate buffered saline (PBS) containing 10% (vol/vol) dimethyl sulfoxide. The tissue fragments were quick frozen, and 40-μm-thick frozen sections were prepared by using a Bright cryostat equipped with a tungsten carbide knife. The sections were pretreated with 0.01% H_2O_2, incubated overnight in PBS/bovine serum albumin containing either anti-fusion mAbs (10C4, 10C5, 10B11, or 12D6) or mouse IgG1, and then incubated for 2 h with peroxidase-conjugated sheep anti-mouse Fab, as described (8). The sections were subsequently processed as described (8). The sections of bone particles and bone tissue were decalcified overnight in 0.1 M cacodylate buffer containing 5% (wt/vol) sucrose and 4.4% (wt/vol) EDTA, pH 7.4. All sections were individually embedded in Epon (Polybed 812). The 1-μm-thick sections were stained with methylene blue.

(*ii*) Tissues were quick frozen, and 6-μm-thick frozen sections were prepared using a Reichert–Jung cryostat (2800 Frigocut). The sections were first incubated overnight in PBS/5% (wt/vol) nonfat dry milk (Carnation, Los Angeles), then incubated for 2 h in PBS/5% nonfat dry milk containing either anti-fusion mAbs (10C4, 10C5, 10B11, or 12D6) or mouse IgG1. Sections were then incubated for 1 h in PBS/5% nonfat dry milk containing a 1:400 dilution of goat anti-mouse Cy3-$F(ab')_2$. Following three washes of 10 min each with PBS, the sections were examined by using the Cy3 excitation filter block (excitation at 550 nm) on an Olympus (New Hyde Park, NY) microscope equipped with UV light.

Cells were cultured on glass coverslips for the indicated time in MEME containing 5% (vol/vol) human serum, fixed in 4% formaldehyde for 1 h at 4°C, and washed for 60 min in PBS/10% fetal calf serum (FCS). Cells were incubated overnight in PBS/10% FCS alone or supplemented with either anti-fusion mAbs (10C4, 10C5, 10B11, or 12D6) or mouse IgG1. Following four washes of 15 min each in PBS/10% FCS, the cells were incubated for an additional 1 h with either FITC- or Cy3-conjugated $F(ab')_2$ goat anti-mouse IgG (1:100 and 1:400 dilutions, respectively) in the same buffer. The cells were examined at either 488 nm or 550 nm by using either the FITC or the Cy3 excitation filter blocks, respectively, on either an Olympus microscope equipped with UV light or a Zeiss Axiovert confocal microscope equipped with a confocal Bio-Rad MRC600 CSLM (Bio-Rad).

Immunoprecipitation and SDS/PAGE Analysis of Cellular Antigens Following Either Metabolic Labeling with [^{35}S]Methionine or Cell Surface Iodination. Rat alveolar macrophages were collected, plated in 6-well plastic dishes at 1×10^7 cells per ml, and cultured in MEME supplemented with 5% (vol/vol) human serum for the indicated times. The cells were either metabolically labeled or surface iodinated as described (12). The labeled cells were scraped and subjected to immunoprecipitation as described (12). In brief, the postnuclear supernatants were incubated successively for 1 h with either anti-fusion mAbs (10C4, 10C5, 10B11, or 12D6) or mouse IgG1 (20 μg/ml), then with goat anti-mouse IgG and *Staphyloccocus aureus* (Zyzorbin, Zymed). The immunoprecipitates were analyzed by electrophoresis on SDS/10% polyacrylamide gels (14).

RESULTS

mAb 12D6 Inhibits Alveolar Macrophage Fusion *in Vitro* and Recognizes a Cell Surface Determinant. To identify putative surface proteins associated with the adhesion and/or the fusion process of macrophages, we set out to generate mouse mAbs which alter the fusion of rat macrophages *in vitro* and which recognize surface determinants. Importantly, all hybridoma supernatants that demonstrated activity (12 of 950 wells) did so by blocking fusion but not aggregation. To ensure that the hybridoma supernatants contained immunoglobulins that interacted with surface determinants, immunolocalization studies were performed with FITC-conjugated goat Fab fragments directed against Fab domains of mouse IgG. Four hybridomas (10C4, 10C5, 10B11, and 12D6) were selected for producing IgG with dual positivity. As a control, mouse IgG1 directed against rat CD4 added to the alveolar macrophages (20–1000 μg) at the time of plating failed to alter the fusion process (Fig. 1*A*). Similarly, the addition of mouse IgG1 mAbs directed against either rat CD44 or rat class II major histocompatibility complex (20–200 μg/ml each) to the fusing macrophages failed to alter fusion (data not shown).

In the presence of mAb 12D6, macrophages induced to fuse aggregated and piled up but failed to fuse (Fig. 1*C*) and to exhibit the smooth surface of multinucleated macrophages (Fig. 1*A*). Although fusion was evaluated morphologically, the blocking effect of mAb 12D6 was estimated to be maximal when added to the macrophages induced to fuse at the concentration of 20 μg/ml. The blocking effect of mAb 12D6 decreased proportionally with the lowering of its concentration to eventually fail to block fusion at the concentration of 0.1 μg/ml. The anti-fusion activity of mAb 12D6 was further demonstrated by prelabeling the macrophages with the lipophilic fluorescent dye $DiOC_{16}$, which incorporates into lipid bilayers and labels membranes (Fig. 1). In the presence of mAb 12D6, aggregated macrophages remained individually labeled (Fig. 1*D*). In contrast, in the presence of mAb W3/25 directed against rat CD4, this fluorochrome concentrated in the center of giant cells where intracellular membranes and nuclei accumulate (Fig. 1*B*).

When fusing macrophages were reacted with mAb 12D6 and analyzed by indirect immunofluorescence using confocal microscopy, a strong membrane signal was detected on the cells that were mononucleated and therefore not yet fused but closely associated with giant cells (Fig. 2). This fluorescent signal did not display the same intensity in every cell and was not regularly distributed on the membrane. It even appeared that a number of cells lacked signal on one segment of their

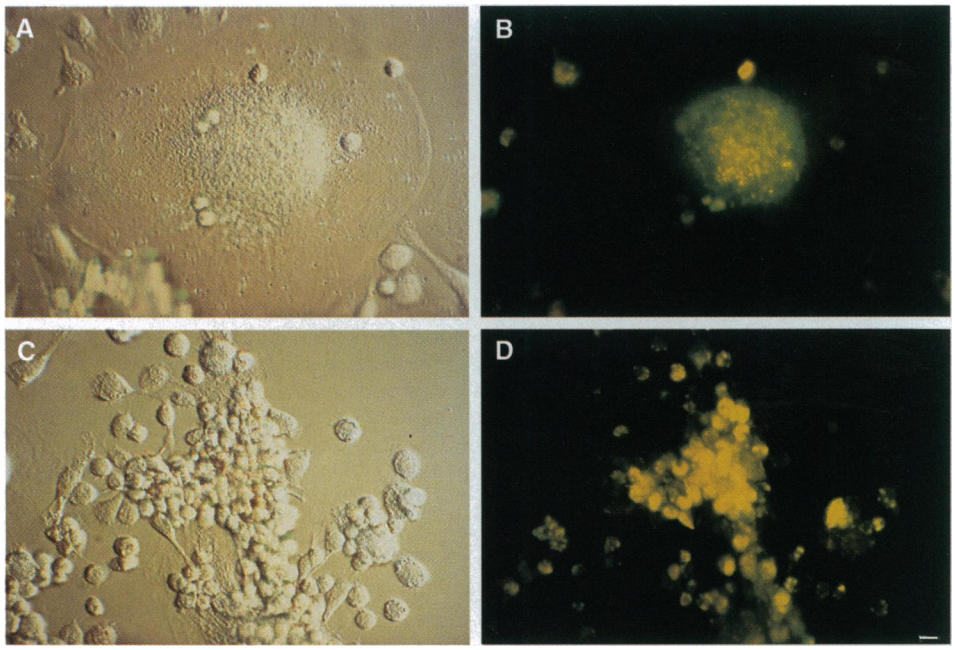

FIG. 1. mAb 12D6 inhibits the fusion of macrophages *in vitro*. Alveolar macrophages were labeled with the lipophilic fluorochrome $DiOC_{16}$ prior to being plated in 6-well dishes at 10^7 cells per ml in medium supplemented with 10% (vol/vol) human serum. Macrophages were cultured for 5 days in medium supplemented with 5% (vol/vol) human serum and 20 μg of either mAb W3/25 (*A* and *C*) or 12D6 (*B* and *D*) per ml. (*A*) Macrophages aggregate and fuse to form multinucleated giant cells which contain hundreds of nuclei. Note the smooth surface and the extensive adherent plasma membrane of the giant cell that is typical of multinucleated macrophages elicited *in vitro*. (*B*) The same giant cell viewed under UV light displays a punctate labeling centrally located around the nuclei. The presence of mAb 12D6 in the culture medium prevents the fusion but not the aggregation of the macrophages (*C*) which remain individually labeled (*D*). (Bar = 10 μm.)

membrane, although that could be due to a lack of access of the antibodies to their antigen. The multinucleated cells failed to display a fluorescent signal. A similar pattern of staining was detected with each of the three mAbs selected which block fusion (data not shown).

To investigate the pattern of expression of 12D6 antigen, alveolar macrophages were plated on glass coverslips in fusogenic conditions and subjected to indirect immunofluorescence at daily intervals after plating. As expected, macrophages had already migrated and aggregated 12 h after plating (Fig. 3*A*). At that time, some of the macrophages already reacted with mAb 12D6. By day 2, when fusion was well underway and multinucleated cells had formed, the mononucleated macrophages appeared larger (about 1.5 times) (Fig. 3*B*). At that time, mAb 12D6 reacted only with mononucleated cells. By day 4, most cells had fused and again only the remaining mononucleated macrophages reacted with mAb 12D6 (Fig. 3*C*). This suggested that during the course of fusion-mediated multinucleation either (*i*) some alveolar macrophages do not express the antigen or else express it in amounts too low to be detected by immunostaining or (*ii*) the antigen undergoes a posttranslational conformational modification induced by culturing in fusogenic conditions that allows the transient exposure of the epitope reacting with mAb 12D6. These data also indicated that the expression of this epitope/antigen is inducible since alveolar macrophages do not express it *in vivo* (data not shown). It therefore appeared that fusion was asynchronous and that successive waves of macrophages expressed 12D6 antigen, apparently preceding fusion. Again, a similar pattern of staining was detected with each of the three mAbs selected for blocking fusion (data not shown).

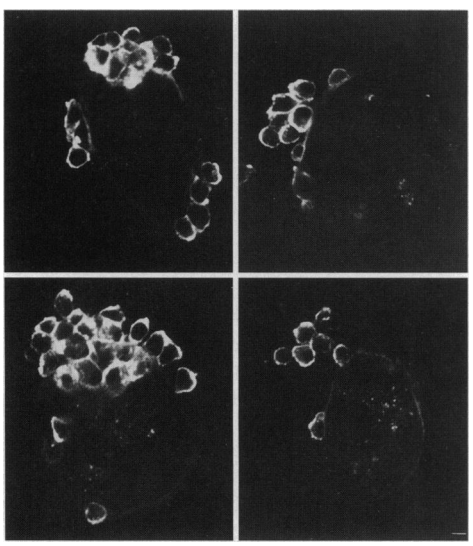

FIG. 2. mAb 12D6 binds a surface determinant expressed on rat alveolar macrophages induced to fuse *in vitro*. Alveolar macrophages were plated on glass coverslips and cultured as described in the legend to Fig. 1 but in the absence of mAb. Cells were fixed after 4 days, a time when fusion is not completed, and stained with mAb 12D6 followed by FITC-conjugated anti-mouse IgG $F(ab')_2$ fragments prior to being subjected to confocal microscopic analysis under UV light. The fluorescent signal is restricted to the surface of the mononucleated cells clustering onto the giant cells shown in the four panels. (Bar = 10 μm.)

mAb 12D6 Recognizes a Surface Determinant on Fusing Macrophages *in Vivo*. To investigate whether the antigen detected by mAb 12D6 was expressed *in vivo* in fusing macrophages and to determine its tissue and cellular distribution, macrophages were induced to fuse *in vivo* by implanting bone particles intramuscularly and subjected to immunolocalization. Sections obtained from bone implants, as well as from bone, bone marrow, brain, liver, spleen, lymph nodes, kidney, lung, skin, striated muscle, and pancreas, were reacted with either mAb 12D6 or mouse IgG1, followed by either sheep anti-mouse IgG Fab fragments coupled to peroxidase (thick frozen sections) or goat anti-mouse IgG $F(ab')_2$ fragments conjugated to Cy3 (thin frozen sections). While each of these tissues

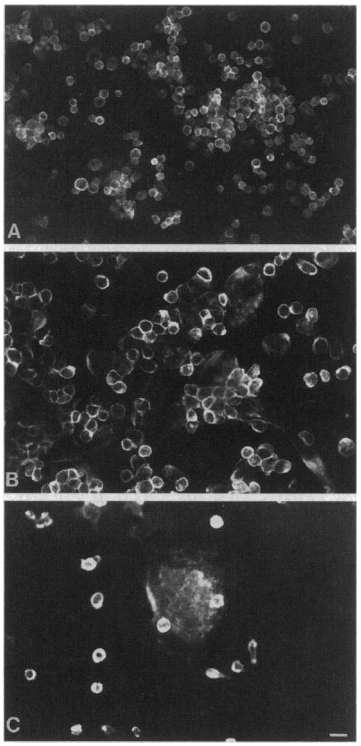

FIG. 3. mAb 12D6 recognizes a molecule whose expression is induced in rat alveolar macrophages by culturing under fusogenic conditions. Cells were isolated, plated on glass coverslips, and cultured under fusogenic conditions for either 12 h (*A*), 2 days (*B*), or 4 days (*C*) prior to being subjected to immunocytochemistry, as described in the legend to Fig. 2 but by using Cy3 as a fluorescent probe. Note the "bouquets" of mononucleated macrophages atop the nonreacting giant cells. *C* was underexposed at the time of printing to improve the visualization of the giant cell. (Bar = 10 μm.)

houses resident macrophages, none of them, including the lungs, reacted with anti-fusion mAb (data not shown). Osteoclasts also failed to react positively. In contrast, cells fusing on implanted bone particles demonstrated a strong signal, indicating that mAb 12D6 recognized a determinant on these cells (Fig. 4 *A* and *C*). The signal appeared to be limited to the closely apposed mononucleated cells which resembled macrophages and to the highly ruffled plasma membrane domain of the multinucleated cells that is located opposite to bone. This is the domain in contact with the incoming and fusing mononucleated cells. The nearby elongated fibroblasts did not exhibit any signal. Indeed, none of the surrounding structures present at this site of chronic inflammation (endothelial cells, etc.) reacted positively (Fig. 4*A*). Sections from implants incubated with mouse IgG1 also failed to demonstrate reactivity (Fig. 4 *C* and *D*). Thus, mAb 12D6 recognizes a surface molecule whose expression is either specific for or enriched in fusing macrophages *in vivo*. Again, a similar signal was detected with each of the three other anti-fusion mAbs that we had selected (data not shown).

mAb 12D6 Precipitates a Newly Synthesized Surface Protein from Fusing Macrophages. Because the mAbs (10C4, 10C5, 10B11, and 12D6) that we generated failed to recognize the denatured form of their antigen, we used immunoprecipitation as a means to analyze these antigens. Fusing alveolar macrophages labeled metabolically with [^{35}S]methionine were subjected to immunoprecipitation with either mAb 12D6 or mouse IgG1. Interestingly, autoradiograms from SDS/PAGE analysis of the immunoprecipitates revealed a diffuse radiolabeled band of 150 kDa suggestive of a highly glycosylated molecule (Fig. 5*A*). When the immunoprecipitation was performed using mAb 10C4, 10C5, or 10B11, a band of similar

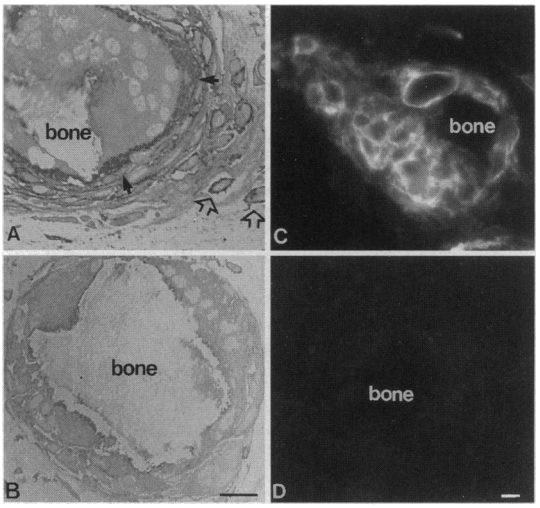

FIG. 4. mAb 12D6 binds a surface determinant expressed on macrophages induced to fuse *in vivo*. Rats were implanted intramuscularly with syngeneic bone particles that were recovered 10 days later and processed for immunolocalization. Frozen sections from bone implants were incubated with either mAb 12D6 (*A* and *C*) or mouse IgG1 (*B* and *D*) followed by either sheep anti-mouse IgG Fab fragments conjugated to peroxidase (*A* and *B*) or goat anti-mouse IgG $F(ab')_2$ fragments conjugated to Cy3 (*C* and *D*). A strong diaminobenzidine reaction product is detected on the surface of large mononucleated cells that resemble macrophages (*A*) (open arrows) while a weaker signal appears on the highly ruffled plasma membrane of the giant cells which surround the bone particles (closed arrows). Note the large number of unstained nuclei accumulated inside the giant cell. A strong fluorescent signal is detected on the surface of the cells that surround the bone particles in thicker sections (6 μm thick in *C* and *D* versus 1 μm thick in *A* and *B*). Note the absence of signal on the plasma membrane domain that faces the bone particles. No reactivity is detected in the presence of mouse IgG1 (*B* and *D*). (Bars = 20 μm.)

mobility was detected (Fig. 5*A*). All additional bands were also detected in the presence of mouse IgG1, indicating that they were nonspecific.

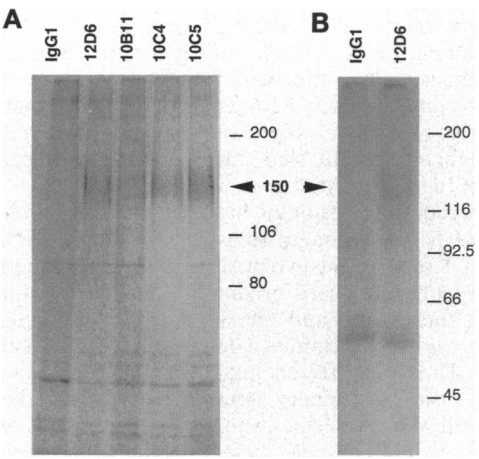

FIG. 5. mAb 12D6 recognizes a 150-kDa surface protein newly synthesized by fusing macrophages. (*A*) Alveolar macrophages were cultured for 24 h in fusogenic conditions prior to being metabolically labeled with [^{35}S]methionine for 17 h and then subjected to immunoprecipitation by using either mouse IgG1, or mAb 12D6, 10C4, 10C5, or 10B11. Immunoprecipitates were analyzed by SDS/PAGE. All four mAbs precipitate a 150-kDa protein. (*B*) Alveolar macrophages were cultured for 4 days in fusogenic conditions, surface iodinated, and subjected to immunoprecipitation with either mouse IgG1 or mAb 12D6. mAb 12D6 precipitates a 150-kDa protein. Immunoprecipitates were analyzed by SDS/PAGE. The mobility of molecular mass standards (in kDa) is indicated. Each lane represents immunoprecipitates from extracts prepared from 1×10^6 plated macrophages.

To confirm biochemically the surface localization of the antigen recognized by mAb 12D6, macrophages were surface iodinated 4 days after being plated in fusogenic conditions and subjected to immunoprecipitation with either mAb 12D6 or mouse IgG1. Autoradiograms from SDS/PAGE analysis of the immunoprecipitates revealed one broad band which exhibited the same gel mobility of 150 kDa as the one previously precipitated (Fig. 5*B*). Two bands of 50 and 90 kDa were detected in the presence of both mAb 12D6 and mouse IgG1 and were nonspecific.

Taken together, these data indicate that all four hybridomas produce antibodies of the IgG1 isotype which block macrophage fusion by virtue of binding to a surface protein. These proteins share a molecular mass of 150 kDa, and are newly synthesized, and their expression is restricted to fusing macrophages and is induced by fusogenic conditions *in vitro* and *in vivo*. The extracellular domain of one of these antigens, 12D6, contains at least one tyrosine residue.

DISCUSSION

By characterizing a highly efficient and pure macrophage fusion assay and by generating mAbs which alter fusion, we have identified a macrophage surface molecule whose expression is restricted to and specific for macrophages that are present in fusogenic environments *in vitro* and *in vivo*. mAb 12D6 blocks macrophage fusion but not aggregation and recognizes a surface antigen whose expression is inducible and transient at the onset of fusion. Taken together, these results suggest that mAb 12D6 recognizes an antigen which potentially plays a role in macrophage fusion.

Interestingly, the three other mAbs selected for blocking fusion and detecting a surface antigen also recognize a newly synthesized protein of 150 kDa. The question as to whether these mAbs recognize the same antigen and/or a conformation epitope remains to be investigated. The fact that all four mAbs detect their antigen transiently at the onset of fusion and fail to recognize their denatured form could suggest that the fusogenic microenvironment induces a transient conformational modification of constitutively expressed molecules. These conformations are sensitive to denaturing conditions and may be implicated in the fusion process.

The cloning of the cDNA coding for this protein(s) will provide the sequence information necessary to express it and study its conformation. Peptide sequence information obtained from the antigen purified by affinity using mAb 10C4 indicates that it does not share identity with known proteins in GenBank (data not shown).

Fusion regulatory proteins have recently been detected in $CD4^+$ U-937 macrophages transfected with the HIV gp160 gene (15). However, the two mAbs that recognize these fusion regulatory proteins were originally selected for stimulating fusion in these cells, and one of these mAbs recognizes a molecule whose N terminus is identical to the $\alpha 3$ subunit of integrins. Thus, this different but interesting approach suggests that the fusion machinery between viruses and host cells includes adhesion molecules whose expression is not restricted to fusing cells. In contrast, the expression of the molecule(s) that we have detected appears to be specific for fusing macrophages. This may be due to the highly efficient fusion assay chosen and the stringent criteria elected for the selection of the mAbs.

The fusion of macrophages is a stepwise process which starts with cell–cell attraction and recognition. We have reported that macrophages fusing *in vivo* display extensive plasma membrane finger-like interdigitations between them, thereby augmenting the extent of their contacting surface (8). This contact may be required for cell–cell fusion. *In vitro*, we have detected the presence of whole macrophages within giant cells (8), suggesting that macrophages may internalize each other. In the present study, the detection of antigen(s) playing a putative role in fusion was restricted to extracellularly located macrophages *in vitro*, although this could be due to a lack of access of the antibodies to intracellular components. Extensive membrane ruffling between fusing cells was confirmed *in vivo*.

The fusion process of macrophages can be conceived as similar to that of myoblasts—i.e., involving a homotypic interaction. This is in contrast with virus–cell and sperm–oocyte fusion, which is heterotypic. Thus, macrophages may express in a mirror-like manner either one or more proteins that mediate or regulate the initial cell–cell fusion event. However, as fusion proceeds, multinucleated cells acquire a new phenotype (8, 10, 11) and fail to express the fusion antigen *in vitro*. Thus, fusion becomes heterotypic between mono- and multinucleated macrophages. Importantly, the fact that mononucleated macrophages fuse with multinucleated macrophages that do not express 12D6 antigen *in vitro* suggests that another molecule(s) is expressed by multinucleated and possibly mononucleated macrophages. Thus, as for virus–cell fusion, macrophages may utilize a "ligand" and a "receptor" to bring about fusion. Although the exact role of the antigen that we have detected in the fusion process cannot be determined at this time, the specificity and the pattern of its expression suggest its possible involvement in fusion and opens possibilities to study the mechanism and/or the regulation of fusion between macrophages and potentially the mechanism of osteoclast differentiation.

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