Vet. Res. 35 (2004) 339–348
© INRA, EDP Sciences, 2004
DOI: 10.1051/vetres:2004017

Original article

Characterization of a novel activation antigen on porcine lymphocytes recognized by monoclonal antibody 5A6/8

Nieves DOMÉNECH[a]*, Rosario BULLIDO[b], Belén ÁLVAREZ[b], Emilia CAMPOS[b], María-Pilar RODRÍGUEZ-CARREÑO[b], Manuel GÓMEZ DEL MORAL[b], Fernando ALONSO[b], Ángel EZQUERRA[b], Javier DOMÍNGUEZ[b]

[a] Laboratory of Investigation, Juan Canalejo Medical Center, Xubias de Arriba, 84, 15006 La Coruña, Spain
[b] Department of Biotechnology, INIA, 28040 Madrid, Spain

(Received 22 December 2003; accepted 19 February 2004)

Abstract – In this report, we describe the characterization of a novel activation antigen on porcine lymphocytes recognized by mAb 5A6/8. This antigen was detected on B and T cells 24 h after treatment with various stimuli. It was also found on alveolar macrophages, and at low levels on untreated monocytes. MAb 5A6/8 precipitated two bands of 45 and 50 kDa under non-reducing conditions, and of 22 and 28 kDa under reducing conditions. The cellular distribution, expression kinetics and/or molecular size of the 5A6/8 antigen differ from those of other known lymphocyte activation antigens. MAb 5A6/8 was able to inhibit lymphocyte proliferative responses driven by different stimuli, suggesting a role for this molecule in the events that lead to lymphocyte activation.

activation antigen / lymphocyte / monoclonal antibody / monocyte / pig

1. INTRODUCTION

Lymphocytes display a large array of surface molecules that vary depending on their maturation, differentiation and activation stage. The characterization of these surface molecules is an important step towards the elucidation of molecular pathways involved in the regulation of immune responses. The activation antigens represent a group of surface molecules whose expression is induced or upregulated during the process of cell activation by an antigen or other stimuli. These molecules participate in different processes associated with cell activation, acting as receptors for cell growth factors, providing co-stimulatory signals or modifying cell adhesion.

In recent years, there has been a growing interest in the immune system of the pig, because of its economical importance as a livestock species and its use as an animal model in biomedical studies. This has led to the production of a large number of monoclonal antibodies (mAbs) against swine lymphocytes [15]. However, in comparison with man and rodents, where well characterized activation antigens have been

* Corresponding author: ndomenech@canalejo.org

assigned to several clusters of differentiation (CD), only CD25 has been defined in the swine [1, 8, 18]. Recently, the porcine CD69 was cloned but no specific mAb is yet available [19].

In this report, we describe the cellular distribution and biochemical properties of a swine activation antigen, identified by mAb 5A6/8. This antigen appears to be distinct from other activation antigens currently characterized in man and rodents.

2. MATERIALS AND METHODS

2.1. Animals and Cells

Large White pigs with an average weight between 30 and 40 kg were used as blood donors. Peripheral blood mononuclear cells (PBMC) were isolated on Percoll discontinuous gradients after blood sedimentation in dextran as has been previously described [6]. The cells were suspended at a density of 10^6/mL in RPMI 1640 medium containing 10% fetal calf serum (FCS), 2 mM L-glutamine, 5×10^{-5} M 2-mercaptoethanol and 30 µg/mL gentamycin (complete medium). For activation, the cells were stimulated with concanavalin A (Con A, Sigma) (2.5 µg/mL) plus PMA (5 ng/mL) for 48–72 h. For immunization purposes, blast cells were obtained by centrifugation for 10 min at 1 500 *g*, over discontinuous Percoll gradients of 70, 50, 40 and 25%. Lymphoblasts were collected from the 50–40% band and washed with PBS. Swine granulocytes, alveolar macrophages, platelets and erythrocytes were obtained as has been previously described [2].

2.2. Monoclonal antibody production

Balb/c mice were immunized by four intravenous injections of about 8×10^6 of pig lymphoblasts. Spleen cells were fused with X63-Ag.8.653 myeloma cells using standard procedures [9] and plated into 96-well microtitre plates. Supernatants were tested for activity against pig lymphoblasts and resting PBMC by flow cytofluorometry. Hybridomas from positive wells were cloned at least twice by limiting dilution. Isotypes of mAbs were determined by ELISA, using rabbit antisera specific for mouse heavy and light chains and a peroxidase-conjugated goat anti-rabbit Ig (Bio-Rad, USA). MAb 5A6/8 was of the IgG_{2a} isotype. MAbs to porcine CD3 (BB23-8E6), CD4 (74-12-4) and CD8 (76-2-11) were kindly provided by M.D. Pescovitz (Indiana University, USA) and J. Lunney (ARS USDA, Belstville, USA), respectively. MAb to porcine CD25 (K231-3B2) and light chains of porcine immunoglobulins (K139-3E1) were kindly provided by C. Stokes (University of Bristol, UK). For labeling, mAbs were purified from ascitic fluid by affinity chromatography on Protein A-Sepharose CL 4B (Pharmacia, Sweeden). Biotinylation of mAbs was performed as previously described [4].

2.3. Immunoprecipitation analysis

Pig lymphoblasts (10^8) were washed three times in PBS and resuspended in 5 mL of PBS. Sulfo-NHS-biotin (Pierce, USA) (0.4 mg/mL, final concentration) was added to the cells and incubated for 15 min at 4 °C. After washing three times with PBS, the cells were lysed with 1 mL of lysis buffer consisting of 10 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP40, 0.2 U/mL aprotinin and 1 mM phenylmethylsulphonylfluoride. The lysate was precleared twice with 50 µL of a 25% (v/v) suspension of protein A-Sepharose (Pharmacia, Sweden) in lysis buffer and then incubated with the different mAbs. Lysate sample (0.1 mL) was added to 0.3 mL of hybridoma supernatant and incubated for 2 h at room temperature with continuous mixing. Then, 40 µL of a 25% (v/v) suspension of Protein A-Sepharose, previously coupled with rabbit anti-mouse Igs, was added and incubated for 1 h. The beads were washed three times with lysis buffer. Immunoprecipitates were finally boiled in electrophoresis

sample buffer with (reduced) or without (unreduced conditions) 2-mercaptoethanol, run in SDS-10% PAGE and transferred to nitrocellulose filters. The filters were incubated with streptavidin-peroxidase (Pierce, USA) and the bands were visualized with the ECL detection assay (Amersham, UK).

2.4. Western blot

Activated cells (5×10^7) were washed twice in PBS and solubilized in 0.5 mL of lysis buffer for 1 h at 4 °C. After centrifugation at 13 000 *g* for 30 min, the supernatant was mixed with the sample buffer and run on SDS-10% PAGE under reducing and non reducing conditions. The separated proteins were transferred to nitrocellulose. Free binding sites on nitrocellulose were blocked with PBS-5% powdered milk. Thereafter, the strips were incubated with hybridoma supernatants, diluted 1/2 in PBS containing 1% BSA and 0.2% Tween 20, for 1 h at room temperature, followed by 1 h incubation with peroxidase-labeled rabbit anti-mouse Ig (Dako, Denmark). Peroxidase activity was visualized using the ECL detection assay (Amersham, UK).

2.5. Flow cytofluorometry

The cells (5×10^5) were incubated on ice with 50 µL of hybridoma supernatant for 30 min. After two washes in PBS containing 0.1% bovine serum albumin (BSA) and 0.01% sodium azide (fluorescence buffer, FB), the cells were incubated for 30 min at 4 °C with 50 µL of FITC-conjugated rabbit $F(ab')_2$ anti-mouse Ig (Dako, Denmark) diluted 1/40 in FB. The cells were washed three times in FB and fixed in 0.3% paraformaldehyde prior to analysis in a FACScan (Becton Dickinson, USA).

For two-color immunofluorescence, the cells were first incubated with mAb 5A6/8 or anti-CD25, followed, after washing, by rabbit $F(ab')_2$ anti-mouse Ig-FITC. Free binding sites were blocked with 5% normal mouse serum. Finally the cells were stained with the biotin-labeled mAbs against porcine CD3, CD4, CD8 or Ig light chains, followed by an incubation with phycoerythrin (PE)-conjugated streptavidin (Southern Biotechnology, USA).

2.6. Induction of antigen expression by different stimuli

PBMC were suspended at 2×10^6 cells/mL, and cultured in the presence of different stimuli: ConA 2.5 µg/mL, PMA 5 ng/mL and/or calcium ionophore A23187 0.5 µg/mL. The cells were harvested after 0 h, 3 h, 24 h, 48 h and 6 days, and the expression of the 5A6/8 antigen was assessed by flow cytofluorometry. Dead cells were excluded by staining with propidium iodide.

2.7. Proliferation assays

PBMC were suspended in complete medium (3×10^5 cells/well, in a final volume of 200 µL) in 96 well flat-bottom microtiter plates (Costar, USA). The cells were cultured for three days in the presence of ConA, at a final concentration of 0.5 µg/mL. For MLR, 1×10^5 irradiated (3 000 rad) allogeneic PBMC were added as the stimulator cells and were cultured for five days. MAbs were added at the initiation of the culture in triplicate. An equal volume of medium was added to the control wells. Cell proliferation was measured by the uptake of ^{3}H-thymidine (0.5 µCi/well) which was added during the final 6 h of culture. The wells were then harvested and assayed for isotope incorporation in an automatic β counter (Wallac, Finland).

3. RESULTS

3.1. Selection of mAb 5A6/8

MAb 5A6/8 was selected by its different reactivity with activated PBMC and resting cells in flow cytometric assays. This antibody recognized a cell-surface antigen present on a high percentage of ConA + PMA activated cells (55–89%), but only, and at lower levels, on a minor subpopulation of resting PBMC (8–17%), identified as

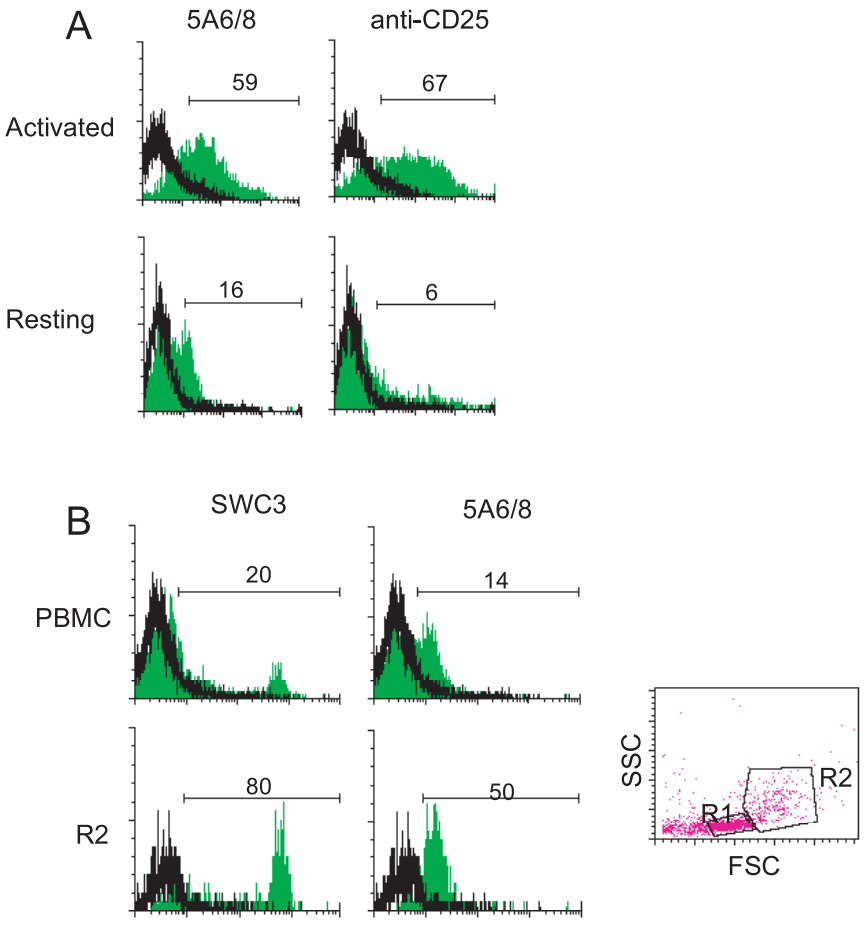

Figure 1. (A) Expression of 5A6/8 and CD25 antigens by resting PBMC and following 72 h of activation with ConA + PMA. **(B)** A comparison of the expression of 5A6/8 antigen with that of the panmyeloid marker SWC3 on PBMC and monocytes (R2), gated according to their FSC and SSC profiles. The open histogram corresponds to background staining with an irrelevant (isotype-matched) mAb. Data from a representative experiment out of three are shown. The numbers indicate the percentage of cells within the histogram regions defined by horizontal bars.

monocytes on the basis of their SSC and FSC characteristics (Fig. 1, R2). The majority of the alveolar macrophages were also clearly stained by mAb 5A6/8 (data not shown). Freshly isolated granulocytes, platelets and erythrocytes were negative. No reactivity was found on PBMC from other animal species (man, horse, cattle and dog), either resting or after activation with ConA plus PMA.

3.2. Biochemical characterization of the 5A6/8 antigen

Immunoprecipitation analyses were carried out with biotinylated lysates of activated PBMC. Under non-reducing conditions, mAb 5A6/8 precipitated two bands of 45 and 50 kDa (Fig. 2A) that upon reduction ran as two bands of 22 and 28 kDa (Fig. 2B), suggesting a disulphide-linked structure.

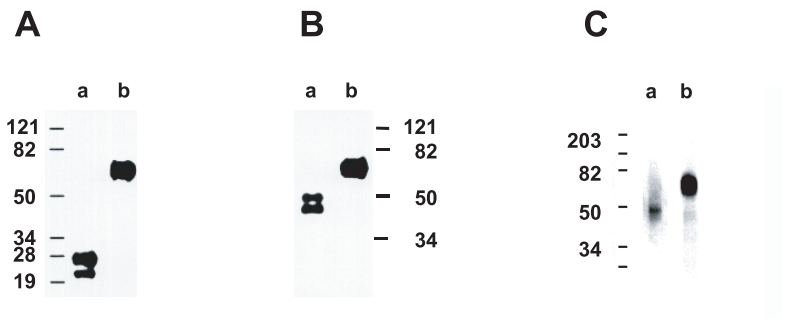

Figure 2. Molecular characterization of the 5A6/8 antigen. **(A)** and **(B)**: Lysates of activated PBMC labeled with biotin, were immunoprecipitated with mAbs 5A6/8 (lane a) or anti-CD25 (lane b) and subjected to SDS-10% PAGE under reducing conditions (A) or non-reducing conditions (B). **(C)** Western blot analysis of lysates from activated blasts under non-reducing conditions, with mAbs 5A6/8 (lane a) or anti-CD25 (lane b). Molecular weight markers are shown at the side of the figures.

Western blot analyses under non reducing conditions also showed also a band of 45–50 kDa (Fig. 2C).

3.3. Expression of 5A6/8 antigen on subpopulations of activated peripheral blood mononuclear cells

The differences in expression of 5A6/8 antigen by distinct PBMC subsets were assessed by two-color flow cytometry on cells incubated with ConA plus PMA (Fig. 3). Most B cells (85% of sIg^+ cells) and T cells (79% of $CD3^+$ cells), either $CD4^+$ (93%) or $CD8^+$ (77%) expressed the 5A6/8 molecule. The majority of these cells were also positive for the CD25 antigen, reflecting their activation stage.

3.4. Induction and kinetics of expression of the 5A6/8 antigen

The capacity of the different stimuli to induce the expression of 5A6/8 antigen was analyzed by flow cytometry at different times after activation. The expression of the 5A6/8 antigen, like that of CD25, was clearly upregulated 24 h after treatment with ConA, PMA, calcium ionophore A23187, or PMA plus A23187, became maximal at 48 h and declined thereafter, except for PMA, with which high levels of expression were still observed at 144 h (Fig. 4).

3.5. Effect of 5A6/8 mAb on lymphocyte proliferation

Functional experiments were carried out to assess whether the binding of 5A6/8 mAb was able to affect cell activation. Various amounts of 5A6/8 mAb, ranging from 0.05 to 5 μg/mL, were added to PBMC cultures that were stimulated with allogeneic cells in an MLR or suboptimal doses of ConA. As shown in Figure 5, mAb 5A6/8 clearly inhibited in a dose-dependent manner, the allogeneic proliferative response and to a lesser extent the proliferation induced by ConA. Both proliferative responses were similarly inhibited by anti-CD25 mAb.

4. DISCUSSION

Few reagents are presently available for the detection of swine activated leukocytes. So far, only two mAbs reactive with the α chain of the IL-2 receptor (CD25) have been characterized [1, 15]. In this report we describe the identification and preliminary characterization of a novel porcine activation antigen, defined by mAb 5A6/8.

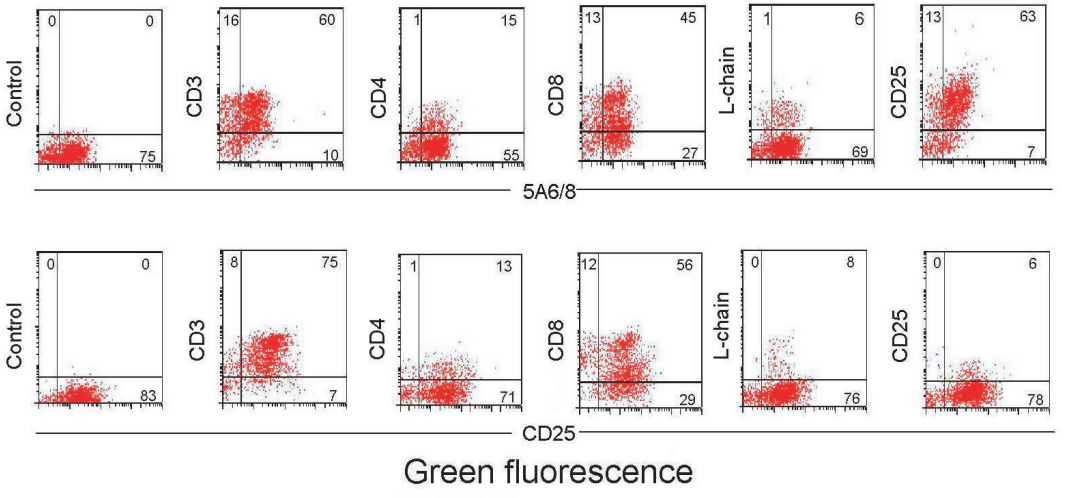

Figure 3. Two-color flow cytometric analysis of 5A6/8 antigen expression vs. the expression of other porcine leukocyte markers on activated PBMC. PBMC were stimulated with ConA plus PMA for 72 h and stained with mAb 5A6/8 or anti-CD25 mAb K231-3B2 and FITC rabbit anti-mouse Ig (green fluorescence), followed by the second biotinylated mAbs (anti-CD3, BB23-8E6; anti-CD4, 74-12-4; anti-CD8, 76-2-11; anti-Ig light chains, K139-3E1; or anti-CD25) and streptavidin-PE. Quadrants were defined by background staining with fluorescent conjugates alone. The numbers in each quadrant represent percentages of cells. The results shown are from one representative experiment of three independently performed experiments (see www.edpsciences.org/vetres for a color version of this figure).

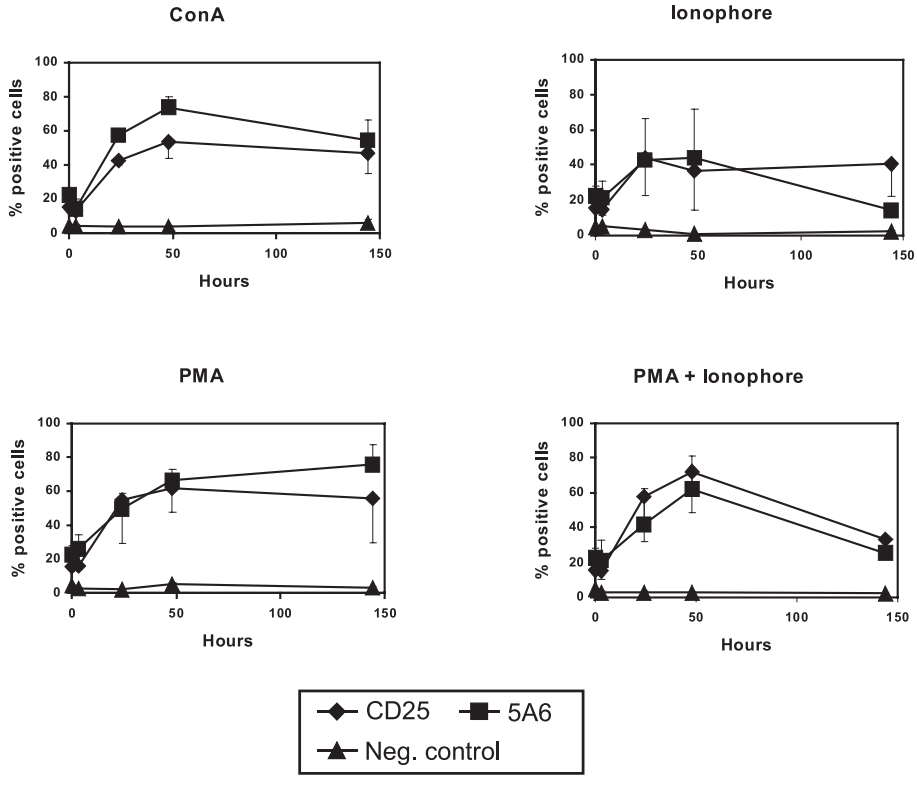

Figure 4. Kinetics of expression of 5A6/8 and CD25 antigens on PBMC activated with ConA (2.5 μg/mL), PMA (5 ng/mL), Ca^{2+} ionophore A23187 (0.5 μg/mL), or PMA (5 ng/mL) + Ca ionophore (0.5 μg/mL). The expression of the antigens was assessed by flow cytometry at different times. The values correspond to the mean of percentages of positive cells, of at least three independent experiments. An irrelevant antibody was used as the negative control.

The antigen identified as mAb 5A6/8 was detectable at low levels on a small percentage of PBMC, but its expression was up-regulated after treatment with PMA or Con A, such that a high proportion of cells expressed this molecule after 24 h. This antigen was observed on a subpopulation of freshly isolated monocytes, and was present, at higher levels, on alveolar macrophages. Although it is possible that the expression of this molecule increases along the maturation pathway of the monocyte to the macrophage, no clear upregulation was observed after the in vitro maturation of macrophages in the presence of porcine serum (data not shown). Alternatively, the high expression found in alveolar macrophages may reflect an activated state of these cells, whose are directly exposed to exogenous materials. MAb 5A6/8 immunoprecipitated two bands of 45 and 50 kDa under non-reducing conditions, that became bands of 22 and 28 kDa under reduction, indicating the existence of dimeric disulphide-bound structures probably consisting of heterodimers and homodimers. The two bands immunoprecipitated by mAb 5A6/8 under non-reducing conditions might also correspond

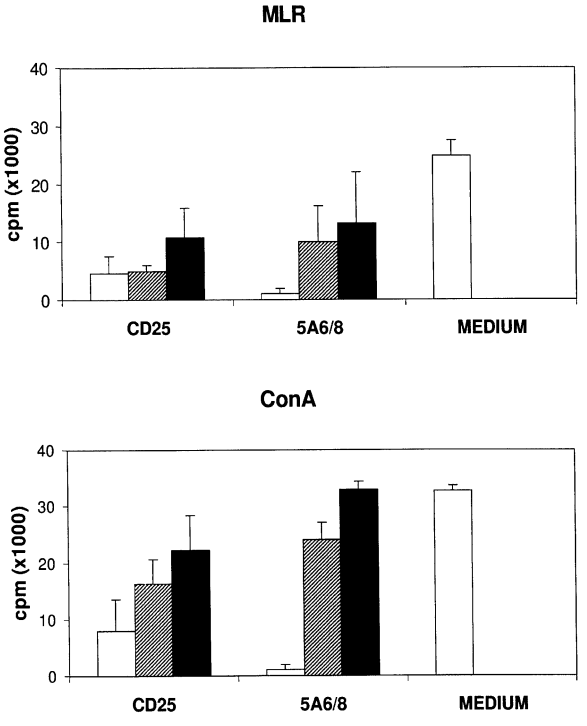

Figure 5. The effect of 5A6/8 and anti-CD25 mAbs on the proliferation of swine PBMC. PBMC (3×10^5 cells/well) were cultured with allogeneic PBMC (1×10^5 cells/well) or ConA (0.5 μg/mL) in the presence of different concentrations of mAbs (open bars, 5 μg/mL; hatched bars, 0.5 μg/mL; filled bars, 0.05 μg/mL). MAbs were added at the beginning of the cultures. [^{3}H]-Thymidine incorporation was measured in triplicate after 5 days (MLR) or 3 days (ConA). Data from a representative experiment out of three are shown. The medium shows the proliferative response in the absence of antibodies.

to different isoforms, as a result of the differences in glycosylation between the different cell lineages, or in phosphorylation corresponding to the different states of activation. These differences have been observed in other antigens such as CD69, CD147 or LPAP [7, 13, 16]. Additional studies, such as treatment with glycosidases or peptide mapping will help to solve these questions.

The 5A6/8 antigen is clearly distinct from most well-characterized human or rodent leukocyte antigens whose expression is upregulated upon activation. It is different from CD25, CD26, CD30, CD70, CD71, CD95, CD96, CD97, CD178, CD223, CD226, CD229, CD245 and CD247 on the basis of both cellular expression and molecular weight [11]. The size and structure, as well as the expression of this antigen by a high percentage of activated cells partially resemble those of CD69, which consists of two polypeptides with molecular weight of 33 and 27 kDa under reducing conditions. However, the activation requirements and the kinetics of expression of the 5A6/8 antigen are significantly different from those of CD69 [3]. Thus, CD69 expression is already detected 2–4 h after activation, peaks at 24 h

and then gradually declines; whereas 5A6/8 was not detected before 24 h, peaked at 48–72 h, and was still detectable after 144 h. On the contrary, the Ca^{2+} ionophore alone was able to induce the expression of 5A6/8 but not that of the CD69 antigen. Another potential candidate for the 5A6/8 antigen is CDw137, a 30 kDa glycoprotein expressed on activated human lymphocytes and monocytes that forms dimers of 55 kDa [14, 17]. However, in this case both the monomer and the dimer can be detected on the cell surface. Another possibility to be considered is that this mAb recognizes a neoepitope on a surface molecule already expressed by resting cells, which results from conformational changes following activation. Expression of new epitopes after activation have been reported, among others, in CD2, CD45 and $\alpha_M\beta_2$ integrin [5, 10,12].

Most of the activation antigens characterized so far have been shown to play relevant roles in the induction and regulation of the cellular events that follow activation. This appears to be true for 5A6/8 since the addition of mAb 5A6/8 to lymphocyte cultures had an inhibitory effect on the proliferative response to alloantigens.

In summary, we have characterized a monoclonal antibody against a new activation marker of porcine lymphocytes that could represent a valuable reagent for studies on the porcine immune system.

ACKNOWLEDGEMENTS

This work was supported by grants BIO2000-1636 and PETRI 95-0573-OP.

REFERENCES

[1] Bailey M., Stevens K., Bland P.W., Stokes C.R., A monoclonal antibody recognising an epitope associated with pig interleukin-2 receptors, J. Immunol. Methods 153 (1992) 85–91.

[2] Bullido R., Alonso F., Gómez del Moral M., Ezquerra A., Alvarez B., Ortuño E., Domínguez J., Monoclonal antibody 2F4/11 recognizes the α chain of a porcine β_2 integrin involved in adhesion and complement mediated phagocytosis, J. Immunol. Methods 195 (1996) 125–134.

[3] Cebrián M., Yagüe E., Rincón M., Lopez-Botet M., de Landazuri M.O., Sánchez-Madrid F., Triggering of T cell proliferation through AIM, an activation inducer molecule expressed on activated human lymphocytes, J. Exp. Med. 168 (1988) 1621–1637.

[4] Dominguez J., Hedrick R.P., Sánchez-Vizcaíno J.M., Use of monoclonal antibodies for detection of infectious pancreatic necrosis virus by the enzyme-linked immunosorbent assay, Dis. Aquat. Org. 8 (1990) 157–163.

[5] Elemer G.S., Edgington T.S., Monoclonal antibody to an activation neoepitope of $\alpha_M\beta_2$ functions, J. Immunol. 152 (1994) 5836–5844.

[6] Gonzalez S., Mendoza C., Sánchez-Vizcaíno J.M., Alonso F., Inhibitory effect of African swine fever virus on lectin-dependent swine lymphocyte proliferation, Vet. Immunol. Immunopathol. 26 (1990) 71–80.

[7] Hamann J., Fiebig H., Strauss M., Expression cloning of the early activation antigen CD69, a type II integral membrane protein with a C-type lectin domain, J. Immunol. 150 (1993) 4920–4927.

[8] Haverson K., Saalmuller A., Chen Z., Huang C.A., Simon A., Seebach J., Boersma W.J., Zwart R., Niewold T.A., Thacker E., Llanes D., de la Lastra J.M., Engelhardt H., Ezquerra A., Alonso F., Dominguez J., Ledbetter J.A., Grosmaire L., Lee R., Nielsen J., Salmon H., Valpotic I., Sver L., Lackovic G., Summerfield A., Khanna K.V., Summary of the first round analyses of the Third International Workshop on Swine Leukocyte Differentiation Antigens, Vet. Immunol. Immunopathol. 80 (2001) 25–34.

[9] Kohler G., Milstein C., Continuous cultures of fused cells secreting antibody of predefined specificity, Nature 256 (1975) 495–497.

[10] Lefrancois L., Bevan M.J., Functional modifications of cytotoxic T-lymphocyte T-200 glycoprotein recognized by monoclonal antibodies, Nature 314 (1985) 449–451.

[11] Mason D.Y., Andre P., Bensussan A., Buckley C., Civin C., Clark E., de Haas M., Goyert S., Hadam M., Hart D., Horejsi V., Meuer S., Morissey J., Schwartz-Albiez R., Shaw S., Simmons D., Uguccioni M., van der Schoot

E., Viver E., Zola H., CD antigens 2001, Tissue Antigens 58 (2001) 425–430.

[12] Meuer S.C., Hussey R.E., Febbi M., Fox D., Acuto O., Fitzgerald K.A., Hodgdon J.C., Protentis J.P., Schlossman S.F., Reinherz E.L., An alternative pathway of T cell activation: a functional role for the 50 Kd T11 sheep erythrocyte receptor protein, Cell 36 (1984) 897–906.

[13] Nehme C.L., Fayos B.E., Bartles J.R., Distribution of the integral plasma membrane glycoprotein CE9 (MRC OX-47) among rat tissues and its induction by diverse stimuli of metabolic activation, Biochem. J. 310 (1995) 693–698.

[14] Pollok K.E., Kim Y.J., Zhou Z., Hurtado J., Kim K.K., Pickard R.T., Kwon B.S., Inducible T cell antigen 4-1BB. Analysis of expression and function, J. Immunol. 150 (1993) 771–781.

[15] Saalmuller A., Characterization of swine leukocyte differentiation antigens, Immunol. Today 17 (1996) 352–354.

[16] Schraven B., Schoenhaut D., Bruyns E., Koretzky G., Eckerskorn C., Wallich R., Kirchgesnerr H., Sakorafas P., Labkovsky B., Ratnofsky S., Meuer S., LPAP, a novel 32-kDa phosphoprotein that interacts with CD45 in human lymphocytes, J. Biol. Chem. 269 (1994) 29102–29111.

[17] Schwarz H., Valbracht J., Tuckwell J., von Kempis J., Lotz M., ILA, the human 4-1BB homologue, is inducible in lymphoid and other cell lineages, Blood 85 (1995) 1043–1052.

[18] Sinkora J., Rehakova Z., Haverson K., Sinkora M., Domínguez J., Huang C.A., Monoclonal antibodies putatively recognising activation and differentiation antigens, Vet. Immunol. Immunopathol. 80 (2001) 143–164.

[19] Yim D., Sotiriadis J., Kim K.S., Shin S.C., Jie H.B., Rothschild M.F., Kim Y.B., Molecular cloning, expression pattern and chromosomal mapping of pig CD69, Immunogenetics 54 (2002) 276–281.