Analysis of rabbit cell surface (CD) antigens by means of cross-reactive monoclonal antibodies with specificity for cattle CD antigens

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ABSTRACT: Studies that involved testing monoclonal antibodies (mAbs) for cross-species reactivity proved to be efficient for the identification of previously unrecognized antigens in a number of different species. Twenty-six mAbs specific to different bovine CD (cluster defined) antigens (CD9, CD18, CD45R, CD41/61, CD62L, MHC class I and bovine IgG light chain molecule) were assayed for reactivity with rabbit peripheral blood leukocytes. Four of the mAbs recognizing CD9 and CD41/61 were reactive with rabbit platelets or granulocytes. These were investigated further by immunoblotting and immunohistochemical staining. The study identified CD9 and CD41/61 molecules on rabbit cells by mAbs IVA-50 and IVA-38. It showed that IVA-50 is a new valuable CD9 reagent for rabbit immunology which could be used for immunofluorescence staining or ELISA assay, immunohistological and molecular studies of rabbit CD9 antigen. IVA-38 recognizes the CD41/61 on rabbit platelets in indirect immunofluorescence and ELISA assay.

Keywords: blood cells; tissue; CD9; CD41/61

The cell differentiation molecules (CD antigens) are cell surface molecules, whose functions are tightly connected with the immune system (Valentovičová et al., 2005; Simon et al., 2007). As the presence of some CD molecules is restricted to a particular population of immunocompetent cells (leukocytes), they are used for the enumeration of those cell populations and for the assignment of the immune profile of animals. Their absence or malfunction on immune cells could lead to a pathological condition and reduced production in consequence of higher morbidity. This is the well-known case of BLAD in cattle (Bovine Leukocyte Adhesion Deficiency) when the mortality of calves is highly frequent due to the absence of molecule CD18 on leukocytes (Kehrli et al., 1990). The study of CD antigens has been concentrated on the cells of man and laboratory animals. The identification of CD molecules of domestic animals reached a different level. Cattle being the species where the CD antigens were analysed by a set of highly specific monoclonal antibodies produced against bovine

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cells by several laboratories and 36 CD specificities may be distinguished by these antibodies (Naessens et al., 1997). The rabbit is farmed mainly for livestock production, but very often it is used as a model animal in biomedical research. Production of mAbs specific of rabbit cells has been sporadic (Wilkinson et al., 1992). Therefore, the study of immune profile and the function of immune system of rabbit are hampered by the lack of rabbit CD reactive mAbs. The aim of this work was the screening of the well characterized bovine CD reactive mAbs for serological cross reactions on rabbit cells in order to find the mAbs which may be used for detection of the homologue molecule on rabbit cells.

MATERIAL AND METHODS

Animals, blood, tissue samples

Eight rabbits of New Zealand breed, used in these experiments, were randomly selected outbred animals that were bred and kept in the Research Institute for Animal Production Nitra. Blood samples were collected with drawn blood from the ear vein of the rabbit and tissue samples after killing the animals. Blood and tissue samples from eight head of cattle were obtained at the local abattoir immediately after the animals were killed.

Isolation of blood cells

Blood samples collected into heparin containing flasks (10 UI/ml) were centrifuged via Verografin (SPOFA, Slovakia) density gradient (Simon et al., 1991) to isolate a different population of blood cells. Mononuclear cells (lymphocytes) were recovered from the interphase, granulocytes from the sediment (after lysis the erythrocytes with Tris-NH_4Cl) and the platelets from the upper plasma layer. The purity of isolated cells was controlled with staining the smears of cells by the Pappenheim method. The purity of each sample was not lower than 95%.

Monoclonal antibodies

Hybridoma cell lines producing mAbs recognizing the bovine cell surface molecules were obtained after immunisation of BALB/c mice with bovine peripheral blood leukocytes using standard procedures for the fusion of SP2/0 with splenocytes of immunised mice, selection and cloning of hybridomas (Kováčiková et al., 2001). The specificity of the majority of tested antibodies was verified in tests performed at the 3rd Workshop on Ruminant Leukocyte Antigens (Naessens et al., 1997). The reactivity of two mAbs detecting MHC class I antigen and light chain of bovine immunoglobulin was analysed separately (Antalíková et al., 2004, 2006). Twenty-six mAbs recognizing CD9, CD18, CD45R, CD41/61, CD62L, MHC class I and bovine IgG light chain molecules were included in the study while some of the assayed antigens were detected with two or more mAbs.

Immunohistochemical staining

For histochemical studies, series of cryostat 5 to 10 μm thick tissue sections were prepared from each sample of rabbit and bovine tissues with Cryocut 1800 (LEICA, Germany). The sections were fixed for 5 min in cold ethanol-acetone mixture (1:1), air-dried, treated with 0.6% H_2O_2 in PBS and then stained using an indirect immunoperoxidase test (Nakane and Pierce, 1967). The sections were incubated with the primary antibody for 45 min at 20°C. The slides were washed with PBS and then incubated for 45 min with peroxidase-conjugated swine anti-mouse Ig (SEVAC, Prague) diluted 1:50 in PBS containing 5% normal bovine serum. After repeated washing in PBS, the sections were incubated in 0.06% (w/v) diaminobenzidine tetrachloride (Sigma, USA) in PBS containing 0.05% (v/v) H_2O_2 for 10 min at room temperature. After washing, the slides were slightly contra stained by Harris's haematoxylin.

Indirect immunofluorescence assay

Indirect immunofluorescence assay described by Boucheix et al. (1983) was used to test the binding of mAbs to blood leukocytes. Briefly, the separated cells were washed three times with PBS with 1% bovine serum albumin and 0.1% sodium azide. The cell suspensions were incubated with monoclonal antibodies in round-bottomed 96-well polystyrene plates for 45 minutes at room temperature. Then the cells were washed 3 times and treated with diluted (1:20–30) fluorescein isothiocyanate (FITC)-conjugated swine anti-mouse

immunoglobulin (SEVAC, Prague) for 45 minutes at room temperature. After further washing the cell suspensions were placed on slides and examined under an epifluorescence microscope (Jenalumar, Zeiss, Germany).

Enzyme-linked immunosorbent assay (ELISA)

ELISA was performed as previously described (Kennett, 1983). The wells in Terasaki microtitre plates were coated with poly-L-lysine and left overnight at 4°C. The plates were three times washed with the solution of 0.9% NaCl. To each well 10 µl suspension of blood cells ($3–4 \times 10^6$ per ml) in phosphate buffered saline (PBS) was added and incubated 2 hours at laboratory temperature. The next step was the incubation of plates with PBS containing glutaraldehyde (20 µl/10 ml PBS) for five minutes. Wells were washed with 0.25% Tween 20 in 0.9% NaCl and twice with 0.9% NaCl, then open sites of the plate were blocked with 0.1% gelatine in PBS for 1 hour at 37°C. After washing three times with NaCl-Tween and twice with NaCl, the aliquots of mAbs were added to each well and the plates were incubated for 2 hours at room temperature. The plates were washed again with NaCl-Tween and NaCl. Swine anti-mouse secondary antibody conjugated to horseradish peroxidase (1:500, SEVAC Praha) was added and incubated for 1 hour at 37°C. The plates were finally washed with NaCl-Tween and NaCl. The peroxidase activity was detected using o-phenylenediamine.

Immunoblotting

Cross reactivity of mAbs was tested by Western blot in protein extracts of rabbit platelets as well as some rabbit tissues (kidney, small intestine, spleen, liver). Platelets and tissues were solubilised in lysis buffer (25mM Tris-HCL pH 7.4; 154mM NaCl; 5mM EDTA; 1% NP40; 2mM PMSF; cocktail of protease inhibitors), separated by SDS-PAGE (non-reduction conditions) (Laemli, 1970), transferred on PVDF membrane (Towbin et al., 1979) and analysed by immunoblotting with primary mAb (IVA-50, IVA-38) followed by anti-mouse IgG alkaline phosphatase conjugate (Sigma, USA) and detected using BCIP/NBT (Roche, Germany).

Table 1. Reactions of mAbs with blood cells of cattle and rabbit

mAbs	Specificity	Lymphocytes				Granulocytes				Platelets			
		bovine		rabbit		bovine		rabbit		bovine		rabbit	
		IF	E	IF	E	IF (%) mean ± SD	E	IF (%) mean ± SD	E	IF (%) mean ± SD	E	IF (%) mean ± SD	E
IVA-50	CD9	–	–	+–	+–	+ + 85.12 ± 7.49	+ + +	+ + 81.87 ± 6.62	++	+ + + 95.37 ± 5.75	+ + +	+ + 92.12 ± 4.64	–
IVA-31	CD9	+–	–	–	–	+ + * 77.87 ± 4.25	+ +	+ * 60.50 ± 5.09	+	+ + * 4.37 ± 4.86	+ +	+ + * 83.37 ± 8.07	+
IVA-38	CD41/61	–	–	+–	+–	+ 6.75 ± 2.60	+ –	+ + 3.37 ± 1.40	–	+ + 89.62 ± 6.11	+ +	+ – 94.12 ± 3.64	+
IVA-125	CD41/61	–	–	+–	+–	+ + * 2.62 ± 1.40	+ –	+ * 1.25 ± 1.03	+–	+ + * 78.62 ± 6.1	+ +	+ * 68.12 ± 6.15	+ –

IF – indirect immunofluorescence; E – ELISA technique;
intensity of reaction +++ intense; ++ moderate; + weak; +– very slight staining of a portion of cells (background); *$P < 0.05$

Statistical analysis

Student's *t*-test was used to compare the reactions of mAbs on rabbit and bovine cells in indirect immunofluorescence.

RESULTS AND DISCUSSION

The panel of 26 mAbs was screened on rabbit blood cells (lymphocytes, granulocytes and platelets). Twenty-two of the assayed mAbs recognizing CD18, CD45R, CD62L and MHC class I and bovine IgG light chain molecules did not show any reaction on the tested rabbit cells. Four mAbs cross-reacted with rabbit cells and stained some types of rabbit blood cells. These mAbs recognize CD9 and CD41/61 on bovine cells which belong to the dominant cell surface molecules of the platelets. The reactions of four mAbs in indirect immunofluorescence and ELISA tests on three subpopulations of rabbit leukocytes were compared with reactions on the same cell populations on bovine cells (Table 1). Comparison of the staining pattern and intensity of reaction of mAbs revealed the high concordance of IVA-50 on the cells of both species. Intensive reactions were observed with the platelets and less intensive staining on the polymorphonuclear cells and no clear reaction was observed on lymphocytes. The reaction pattern of IVA-31 was similar to that of IVA-50 but the staining was less intensive and a significant difference was found in the distribution of reactions on bovine and rabbit cells. The expression of CD9 on rabbit leukocytes has demonstrated, as expected from the known distribution of human CD9 (Barclay et al., 1997) and the reactions of IVA-50, that CD9 is a major platelet and granulocyte antigen in cattle as well as in rabbits. Similarly to man, CD41/61 antigen recognized by mAbs IVA-38 and IVA-125 is of a restricted distribution being expressed on bovine and rabbit platelets but not on granulocytes or lymphocytes. However, the IVA-125 in rabbit has a weak reaction and there is a significant difference between the staining patterns on bovine and rabbit platelets and this mAb does not cross-react with the homologous CD41/61 molecule on rabbit cells.

To obtain efficient mAbs for detection of homologous molecule in another species a more complex analysis is needed. The minimal requirements (internationally accepted), besides the immunofluorescence staining of cell populations, are the tissue binding properties (immunohistology) of mAbs supplemented by comparable molecular weight estimates of appropriate protein in the heterologous species. In this study, four mAbs were tested simultaneously by immunoblotting and indirect immunoperoxidase staining with tissue samples of selected organs (lymph node, spleen, small and large intestine, kidney, liver, lung, mammary gland) of both species. Reactions of the mAbs IVA-38, IVA-125 and IVA-31 with rabbit tissues were weak and sporadic. Therefore, no appreciable data were obtained from their testing. The Western blot analysis showed that the molecular

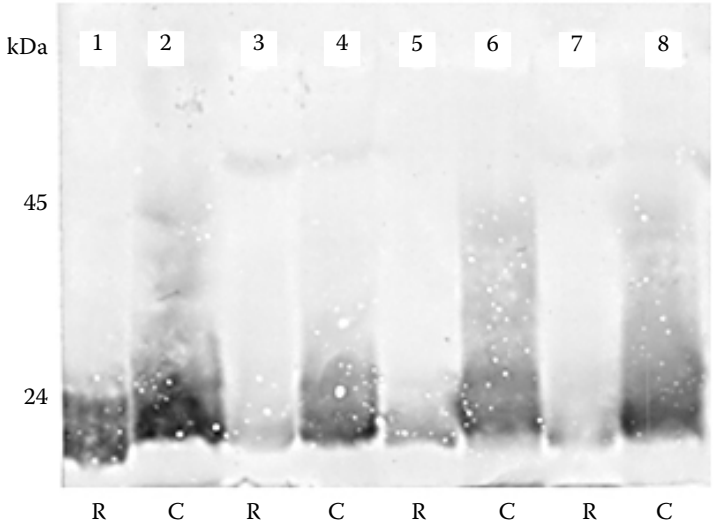

Figure 1. Cross reactivity of IVA-50 with rabbit platelets and tissues
1, 2 – platelets; 3, 4 – kidney; 5, 6 – small intestine; 7, 8 – spleen;
C – cattle; R – rabbit

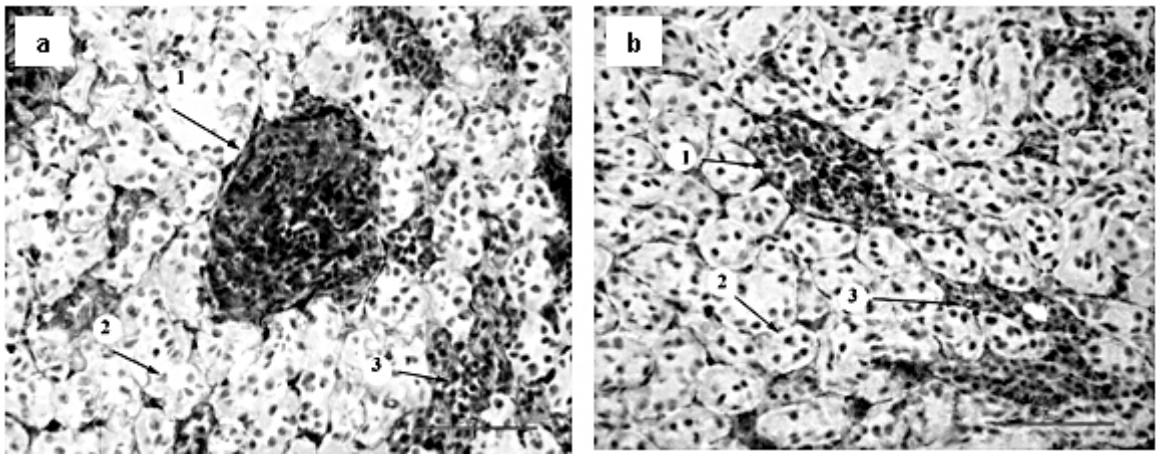

Figure 2. Tissue sections of kidney (cortex)
(a) rabbit; (b) cattle;
1 – nephron (glomerulus and Bowman's capsule); 2 – transversal section of proximal and distal parts of nephron
3 – interstitial tissue

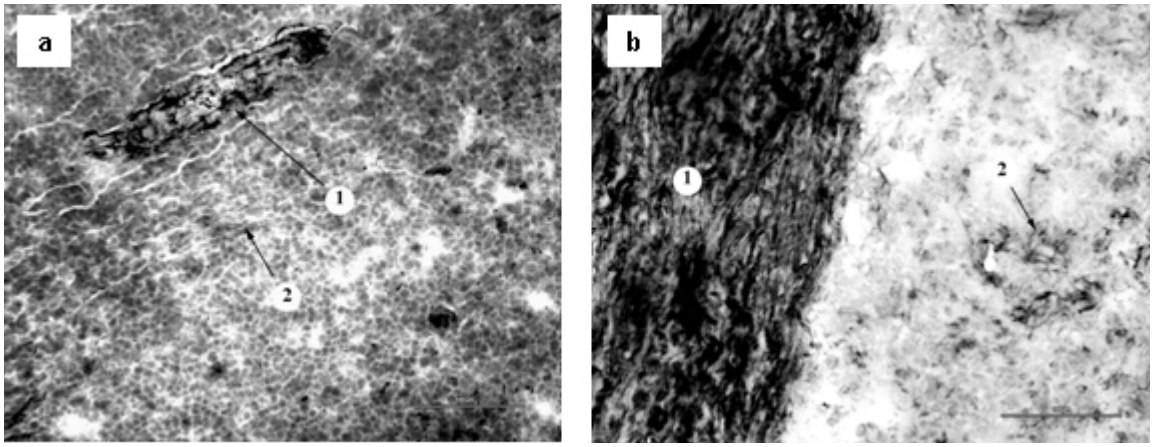

Figure 3. Tissue sections of spleen
(a) rabbit; (b) cattle;
1 – trabeculae of red pulp; 2 – Billroth's cords of red pulp

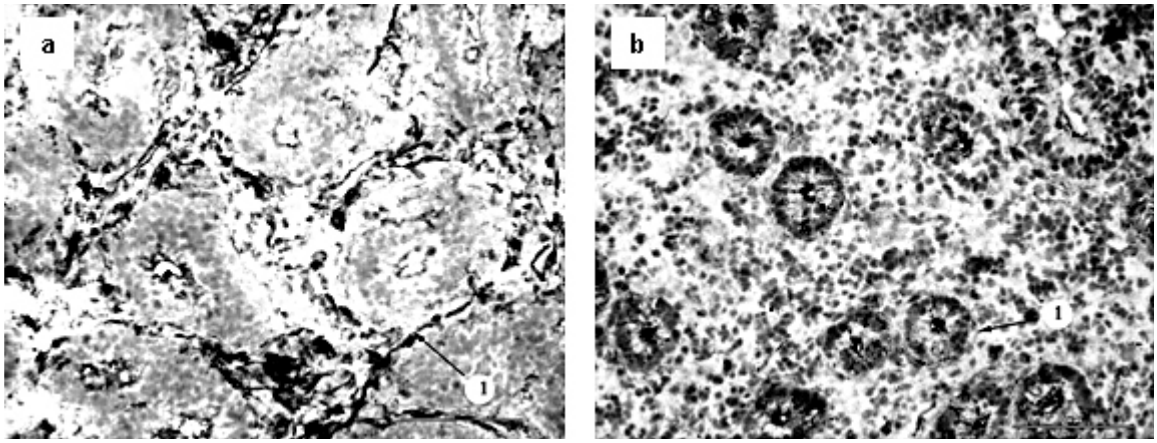

Figure 4. Tissue sections of small intestine
(a) rabbit; (b) cattle; 1 – Lieberkühn's crypts

weight of antigen recognized by IVA-50 on rabbit platelets/tissues is in good agreement with the molecular weight of homologous CD9 molecule. The IVA-50 in all platelets and tissues (kidney, intestine, spleen, liver) protein extracts under non-reduction conditions recognized the band with molecular weight 22 to 24 kDa (Figure 1). Tissue sections from the same organs as for Western blotting were used in immunoperoxidase assay to test the immunoreactivity of IVA-50 in rabbit and bovine tissues. The staining pattern of the cryostat sections of both species was very similar. In the kidney the high expression of CD9 was found in the nephron (glomerulus) and interstitial tissue with lower intensity in rabbit (Figure 2a,b). In the spleen the trabeculae and Billroth's cords of red pulp (Figure 3a,b) and in the intestine the Lieberkühn's crypts were strongly stained (Figure 4a,b). In the liver the Kupffer's cells were reactive.

This study has clearly identified the CD9 molecule on rabbit cells. It has been shown that IVA-50 is a valuable new CD9 reagent for rabbit immunology. It could be used for immunofluorescence staining or ELISA assay, immunohistological and molecular studies of rabbit CD9 antigen. That is not the case of IVA-38, which recognizes the CD41/61 on rabbit platelets in indirect immunofluorescence and ELISA assay but fails to react with the target molecule in body tissues or immunoblotting assay.

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