

Characterization of a bovine leucocyte differentiation antigen of 145,000 MW restricted to B lymphocytes

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SUMMARY

A new bovine B-cell differentiation antigen is described that is detected by three monoclonal antibodies (mAb). The antigen is not an immunoglobulin and is precipitated from peripheral B cells as a molecule with an approximate molecular weight (MW) of 120,000 or 145,000 before and after reduction, respectively. Data obtained from two-colour cytofluorimetry and immunohistochemistry confirmed that the antigen was found only on mature B cells and on cells with dendritic morphology in the follicles of the organized lymphoid tissues. Its level of expression is directly correlated with that of IgM on peripheral blood B cells and *Theileria parva*-transformed B cells. The marker was also expressed on the peripheral cells which expressed surface IgG. Based on the antigen's cellular distribution, biochemistry and histochemistry, it is considered to be analogous to the human CD21 antigen.

INTRODUCTION

Studies of differentiation antigens on human B lymphocytes have shown that these molecules can be useful in defining B-cell maturation stages and in analysing B-cell activation and immunoglobulin secretion. Unfortunately, mouse monoclonal antibodies (mAb) defining human leucocyte surface markers cross-react relatively infrequently with cell-surface antigens in other species (Aasted *et al.*, 1988). Thus, in order to study bovine B-cell differentiation, new mAb have to be generated against bovine B-cell antigens.

A number of mAb that react with bovine B cells have already been generated (Lewin, Davis & Bernoco, 1985; Davis *et al.*, 1987). However, they are either not restricted to the B-cell lineage, for example some are anti-MHC class II, or they react with IgM. Two new mAb against bovine B cells (Kunita, Koyama & Saito, 1988) that detect antigens other than Ig, Fc receptor or complement receptor, are not lineage restricted. mAb BLMo-4 reacts with monocytes and all Ig⁺ cells, while mAb BLMo-10 recognizes the majority of Ig⁺ cells and also a small subpopulation of thymocytes. Not enough information is available to relate them to known human CD antigens.

In this study, the cellular distribution and biochemical characteristics of a bovine B-cell-specific surface antigen and three mAb that react with it are described.

Abbreviations: FITC, fluorescein-isothiocyanate; Ig, immunoglobulin; mAb, monoclonal antibody(ies); MW, molecular weight; PBMC peripheral blood mononuclear cells; PE, R-Phycoerythrin; slg, surface Ig;

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MATERIALS AND METHODS

Production of mAb

One of the hybridomas, originally referred to as mAb B4/27.23.4 (Pinder, Pearson & Roelants, 1980), was produced from spleen cells of BALB/c mice immunized with peripheral blood mononuclear cells (PBMC) from a Friesian bovid by fusion with X63-Ag8 myeloma cells. The hybridoma cells were recloned by limiting dilution, and one positive clone, called IL-A65, was used for production of ascitic fluid.

mAb CC21 was produced from spleen cells of a BALB/c mouse immunized with alveolar macrophages isolated by tracheo-bronchial lavage from a Friesian-cross (*Bos taurus*) calf. mAb CC51 was derived from a mouse immunized with mesenteric lymph node cells from a Friesian cow. Mice were injected twice intraperitoneally with cells and Quil-A adjuvant (Superfos, Vedbaek, Denmark) and once intravenously without adjuvant 4 days prior to fusion. Spleen cells were fused with NS-1 myeloma cells (ATCC, Rockville, MD). Screening, cloning and production of ascitic fluid was as previously described (Howard *et al.*, 1988).

Ig subclasses were determined by fluorimetry using FITC-labelled mouse isotype-specific antibodies (Bionetics, Charleston, SC) or by immunodiffusion with Miles reagents (ICN-Immunobiologicals, High Wycombe, Bucks, U.K.).

Isolation of leucocytes

PBMC and spleen cells were isolated from Boran cattle (*Bos indicus*) as described previously (Naessens *et al.*, 1985). Monocyte-enriched populations were obtained by adherence to

plasma-coated gelatin, as described elsewhere (Goddeeris *et al.*, 1986). PBMC were isolated from Friesian-cross cattle on Ficoll (Histopaque 1083; Sigma, Poole, Dorset, U.K.).

Cell lines

Pokeweed mitogen and Con A-stimulated cells were generated as described previously (Naessens *et al.*, 1985). *Theileria parva*-infected clones of T, B and null cells were produced as described elsewhere (Baldwin *et al.*, 1988a). An IL-2-dependent cloned bovine cell line, T19.4, was obtained from Dr W. I. Morrison, ILRAD.

Other mAb used in assays

mAb IL-A26 and IL-A28, both IgM, recognize the bovine CD2 and CD6 antigens, respectively (Baldwin *et al.*, 1988b). mAb CC42 and IL-A42 detect bovine CD2; both inhibit binding of other anti-CD2 antibodies (Davis *et al.*, 1988). An antigen specific for bovine non-T4, non-T8, non-sIg lymphocytes (γ, δ T cells) was detected by mAb IL-A29 (Morrison *et al.*, 1989). mAb IL-A46 reacts with monocytes and other myeloid cells, but not resting lymphocytes (D. McKeever and W.I. Morrison, personal communication). mAb IL-A30 detects a bovine IgM isotypic determinant and mAb IL-A2 is specific for IgG (Naessens *et al.*, 1988). mAb IL-A58 and IL-A59 are specific for bovine Ig light chains (Williams, Newson & Naessens, 1990) and detect all Ig. mAb J11 detects a monomorphic determinant on bovine class II antigens (Baldwin *et al.*, 1988c). IL-A mAb were produced at ILRAD; CC mAb were produced at Compton Laboratory.

Immunofluorescence

Cells were stained by indirect immunofluorescence (Naessens *et al.*, 1985) and analysed on a fluorescence-activated cell sorter (FACStar PLUS or FACScan; Becton-Dickinson, Sunnyvale, CA). Fluorescence was measured on a log scale; (from 0 to 10,000), in 1024 channels and the mean fluorescence was calculated using the formula:

$$\frac{\sum_i n_i \times 10^{i/256}}{\sum_i n_i}$$

where i is the channel number from 1 to 1024, and n_i is the number of cells in channel i .

For two-colour immunofluorescence the second-step reagents were FITC-labelled (Bionetics or Southern Biotechnology Inc., Birmingham, AL) and biotinylated (Amersham International, Amersham, Bucks, U.K.) isotype-specific antibodies, followed by phycoerythrin-conjugated streptavidin (Becton-Dickinson).

Inhibition of binding

The ability of the three mAb IL-A65, CC21 and CC51 to inhibit the binding of each other was determined by incubating PBMC with one mAb, followed by incubation with a second mAb and then with biotin or FITC-conjugated antiserum specific for the isotype of the second mAb. Controls included similarly incubated PBMC with the first mAb omitted. The ability of mAb IL-A30 to inhibit binding of IL-A65 and CC51 was tested in a similar manner.

Lymphoid tissue staining

Tissues for cryostat sectioning were obtained from slaughtered

animals. Frozen sections of bovine lymph node, thymus, ileal and jejunal Peyer's patches, spleen, tonsil, kidney, liver and skin were prepared and stained by the indirect immunoperoxidase method (MacHugh *et al.*, 1988; Howard *et al.*, 1988).

Biochemical analysis

Immunoprecipitation was performed as described previously (Naessens *et al.*, 1985, 1988). Lymphocytes were surface radioiodinated by the lactoperoxidase technique, lysed, and the lysates were preadsorbed with protein A-Sepharose (Pharmacia, Uppsala, Sweden). Ten microlitres of ascitic fluid were added and 1 hr later precipitated with 50 μ l of packed protein A-Sepharose. For sequential precipitations this was repeated until no more radioactivity was bound. One more step with protein A-Sepharose was done to ensure that all antibody was removed. Precipitation with the second antibody was then performed in the same way as with the first antibody.

Purification of antibodies and coupling to matrix.

Bovine Ig were purified as described previously (Naessens *et al.*, 1988): IgG by anion exchange, IgM on Sephacryl S300 and IgA by affinity chromatography using anti-bovine IgA mAb (Williams *et al.*, 1990). IL-A65 was coupled to CNBr-activated Sepharose (Pharmacia) according to the manufacturer's instructions.

RESULTS

mAb specific for B cells

mAb IL-A65, CC21 and CC51 were of the IgG2a, IgG1 and IgG2b isotypes, respectively. All three mAb were shown to stain a subpopulation of lymphocytes in cell preparations of peripheral blood, lymph node, ileal and jejunal Peyer's patches, tonsil and spleen. Thymocytes were completely negative, as were all bone marrow cells, except for the few Ig⁺ cells that were found in animals over 2 years of age ($\leq 3\%$). Percentages of positive cells in the blood varied according to the animal, but were not significantly different from the number of Ig⁺ cells. Table 1 shows that the percentages of cells positive for the different mAb in PBMC and tonsil were the same as the percentage of IgM⁺ cells. When the anti-IgM antibody IL-A30, together with each of the new mAb, was added to the lymphocyte preparations, the percentage of positive cells did not increase, indicating that the mAb detect the same subpopulation as IL-A30 and that all three of the new mAb were detecting the same subpopulation.

Cells from lymph nodes stained with the mAb fluoresced at a higher intensity than the B cells from peripheral blood or Peyer's patches (Fig. 1).

Inhibition of binding

To check whether all three mAb detected the same or different epitopes, inhibition studies were performed on the binding of each mAb to the target antigen by the other mAb. None of the mAb inhibited the binding of the other two, suggesting that mAb recognized different epitopes. Similarly, inhibition was not observed with anti-Ig antibodies, whether monoclonal (Williams *et al.*, 1990) or polyclonal (rabbit anti-bovine IgG or IgM).

Table 1. Percentage of cells stained with the different mAb

mAb used for staining (specificity)	Cell source*	
	PBMC	Tonsil
CC42 (CD2)	52	ND
IL-A42 (CD2)	ND	50
IL-A30 (IgM)	18	34
IL-A65	17	38
CC21	16	34
CC51	18	37
IL-A30+IL-A65	21	37
IL-A30+CC21	20	38
IL-A30+CC51	22	38
CC21+CC51	19	ND
CC42+CC51	69	ND
ILA42+CC51	ND	81

*The PBMC and tonsillar cells were each taken from different Friesian calves.
ND, not done.

Staining of tissues

The three mAb (IL-A65, CC21 and CC51) produced a similar staining pattern on all tissue sections examined. In lymph nodes, they stained the B-cell follicles and cells in the interfollicular areas of the cortex (Fig. 2a). These represented probably all of the cells that stained for sIgM in these areas. Cells in the paracortex were not stained. In the follicles, all the cells in the mantle zone were strongly stained and the blast cells in the germinal centre also appeared to be positive. In addition, a dense meshwork pattern of staining was observed in the germinal centres, similar to but more dense than that seen with anti-Ig. This pattern of staining is similar to the staining of human dendritic reticulum cells by CD21 (Ling, Maclennan & Mason, 1987).

In the spleen (Fig. 2b), the three mAb strongly stained cells in the B-cell areas that stained for sIgM. The follicles stained very strongly, while cells in the mantle zone stained more diffusely. Cells in the periarteriolar lymphocytic sheath did not stain.

Cells in the follicles in the tonsil stained strongly with all three mAb. Positive cells appeared to include lymphocytes and cells with dendritic morphology (Fig. 2c). In sections of Peyer's patch taken from the ileum the cells in the follicles stained strongly (Fig. 2d). A high proportion of cells in the dome and some cells in the dome-epithelium also stained. Positive cells in the lamina propria and epithelium of the villus were very infrequent.

In lymphoid tissues binding was only apparent to lymphocytes or cells with reticular structure in or around the follicles. No binding was seen to sections prepared from non-Peyer's patch gut tissue, nor in sections from liver, kidney and skin.

Double staining

T cells, Ig⁺ cells and γ,δ T cells were analysed on a FACStar after double fluorescence staining. Figure 3a shows that mAb IL-A65 does not recognize T cells, which were labelled by a

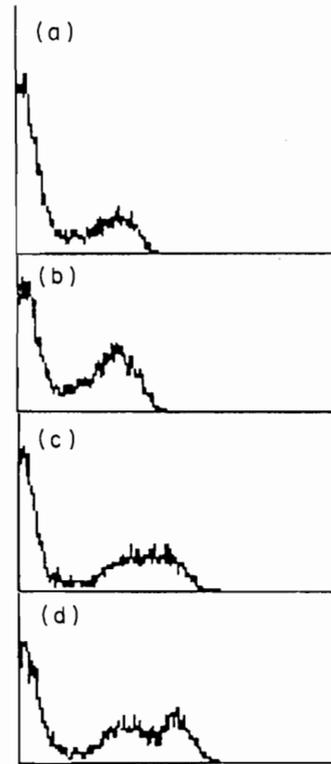


Figure 1. Fluorescence intensities of cells stained with mAb CC51 from (a) PBMC, (b) ileal Peyer's patches, (c) prescapular and (d) preformal lymph nodes. Positive lymph node cells were always brighter than PBMC.

mixture of two anti-T cells mAb directed against bovine CD2 and CD6. Similarly, the antigen was not expressed on γ,δ T cells (Fig. 3b). Identical plots were obtained with mAb CC21 and CC51 (not shown). Peripheral blood monocytes, the purity of which was assessed by ANAE staining (Goddeeris *et al.*, 1986) and by mAb IL-A46, did not stain with mAb IL-A65.

The pattern obtained when co-staining PBMC with anti-Ig was very different. In Fig. 3c, the antigen, here labelled with CC51, and Ig occurred on the same cells. Furthermore, there was a close correlation between level of expression of Ig and level of expression of the antigen recognized by CC51. mAb J11 (anti-MHC class II) also detected all Ig-positive cells (Fig. 3d), but the expression of MHC class II and Ig were not correlated: B cells expressing low amounts of Ig still expressed a high level of class II on their surface. When double staining was performed with anti-IgG on lymph node cells, few IgG cells were present, but those which were present were also positive for the antigen recognized by CC51.

Analysis of bovine B-cell clones

Bovine B-cell leukaemia lines were not available to this study. However, it was possible to obtain continuously growing lines of bovine lymphocytes by infecting them with *Theileria parva*. Although *T. parva*-infected B cells change their surface phenotype (Naessens *et al.*, 1985) and gradually lose their capacity to express IgM, some clones retain IgM on their surface up to 2 months after infection (Baldwin *et al.*, 1988a).

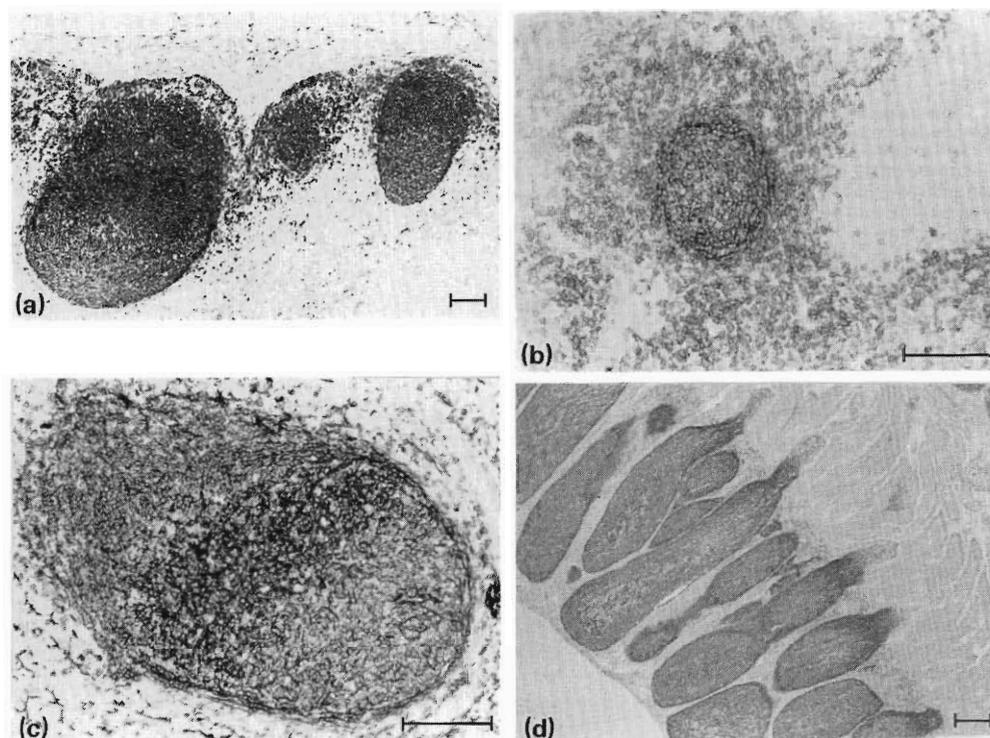


Figure 2. Cryostat sections of (a) lymph node, (b) spleen, (c) tonsil and (d) ileal Peyer's patches, stained with mAb IL-A65 (a), CC51 (b, c) and CC21 (d). Bars represent 200 μ m.

Table 2 summarizes the data obtained when clones of *T. parva*-infected B cells were stained with mAb IL-A65, CC21 and an anti-Ig, IL-A58 (Williams *et al.*, 1990). The mean cell fluorescence is a relative number obtained from the fluorescence histogram by the formula given in the Materials and Methods. Intensity depends not only on the amount of antigen on the cell surface, but also on the affinity of the mAb and the reaction with the second-step antibody. Therefore, one cannot directly compare mean fluorescence of one mAb with another, but one can compare fluorescence intensities of the different B-cell clones for a given mAb. It is striking that clones positive for sIg were also positive for the antigen detected by IL-A65 and CC21, while other clones reacted weakly or not at all with all three mAb. When the mean fluorescence of any two mAb on the B-cell clones was compared using Kendall's rank correlation test, a positive correlation was found at the 5% significance level.

Lack of relationship to serum protein

mAb IL-A65 and CC21 were tested by ELISA (Williams *et al.*, 1990) to determine whether they bound to serum Ig. None of the three mAb bound to purified IgM, IgG or IgA.

Thirty micrograms of purified IL-A65 antibody were coupled to CNBr-activated Sepharose, and 20 ml of serum were passed over it. No serum protein bound specifically to the mAb, indicating that the B-cell antigen is not secreted into the serum.

Immunoprecipitations

PBMC were radiolabelled with 125 I and lysed. Precipitations were carried out with all three mAb and the antigen was

analysed by SDS-PAGE under reducing and non-reducing conditions (Fig. 4). All three mAb revealed the same band with a relative MW of around 120,000 under non-reducing and 145,000 under reducing conditions on a 5–10% gradient gel. Preabsorption with mAb IL-A65 or CC51 removed all antigen precipitable by the other antibody, showing that they detect the same molecule. Also, mAb CC21 failed to precipitate antigen after using mAb IL-A65 to clear the lysate and, therefore, the two mAb detected the same antigen.

Preabsorption of the lysates with antibodies to bovine Ig did not influence the precipitation of the p145 molecule by the mAb.

DISCUSSION

This paper has described three mAb that detect a new bovine B-cell antigen different from Ig or class II MHC. Sequential immunoprecipitations with the mAb revealed that they detect the same molecule of approximately 120,000 MW non-reduced and 145,000 MW after reduction. A small increase in apparent MW after reduction is often observed in SDS-PAGE, because molecules lose their globular conformation by cleavage of internal disulphide bonds and as a result migrate more slowly through the gel. The mAb did not inhibit the binding of one another to the surface of B cells, and therefore recognize different epitopes on the same membrane molecule.

The antigen was absent from thymocytes and from almost all bone marrow cells (only 3% positive). On non-lymphoid tissues (kidney, liver, gut and skin) no staining with the mAb was observed. However, it was present on a subpopulation of cells in PBMC, tonsil and Peyer's patches and was strongly

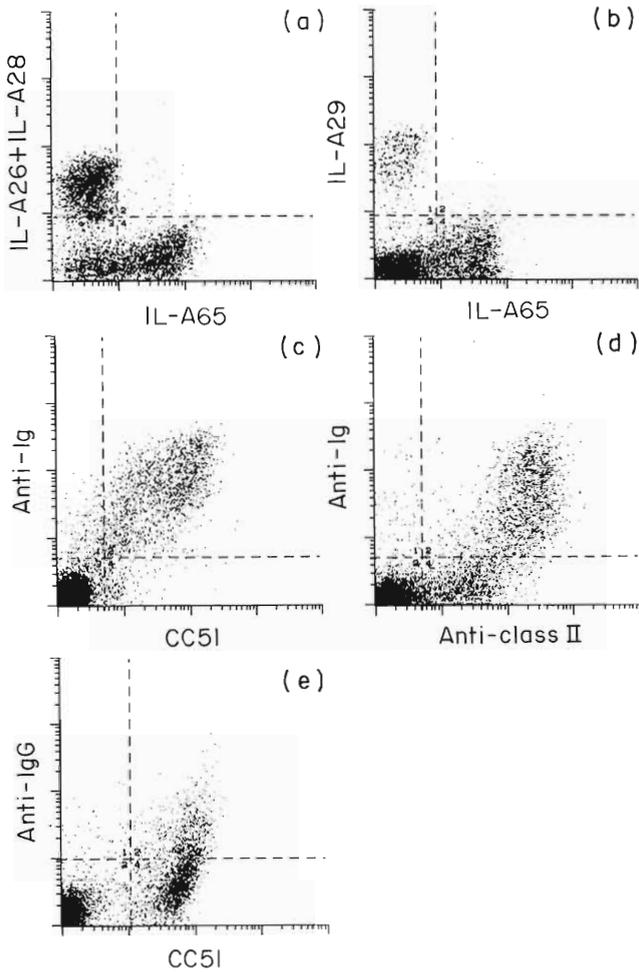


Figure 3. Two-colour fluorescence on bovine PBMC (a-d) or lymph node cells (e). Green fluorescence (FITC) on horizontal axis; red fluorescence (PE) on vertical axis. Comparison of the cell distribution of the B-cell antigen (horizontal axis) with the distribution of T-cell antigens (a), a γ, δ T cell antigen (b), total Ig (c) or IgG (e). Class II and Ig are compared in (d).

Table 2. Relative mean cell fluorescence of *T. parva*-infected B-cell clones stained with different mAb

Clone no.	mAb		
	IL-A58*	IL-A65	CC21
1	6.7	6.4	2.2
2	40.0	117.8	55.2
3	16.1	19.4	7.2
4	27.8	9.7	1.6
5	43.9	89.7	70.7
6	4.9	3.4	2.8
7	3.2	0.15	0.24
8	46.3	86.0	42.6

* Reacts with bovine Ig.

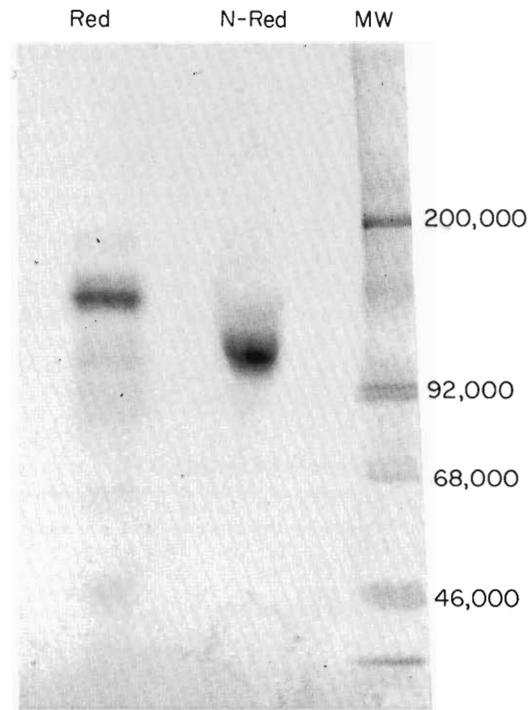


Figure 4. Autoradiography of SDS-PAGE of immune precipitations with mAb IL-A65 under non-reducing (N-Red) or reducing (Red) conditions.

expressed on a population of cells from lymph node. By two-colour fluorimetry using well-defined antibodies, it was shown that the antigen was only present on Ig⁺ cells and not on T cells, γ, δ T cells or cells from the myeloid lineage. Immunohistochemical analysis also demonstrated that cells in the B-cell areas in lymph nodes, spleen, tonsil and Peyer's patch were stained but those in the T-cell areas of these tissues and in the thymus (cortex or medulla) were not. Thus, in the organized lymphoid tissues, the three mAb stained B cells but not T cells. In the lymphoid follicles, cells that had the morphological appearance of dendritic cells were also stained. It is not known whether the dendritic cells expressed the antigen or passively acquired it from other cell types, as hypothesised for human CD21 (Nadler, 1985).

The simultaneous expression of IgM and the p145 antigen on the cell-surface of B cells is unique among bovine cell markers. This correlation was found on normal PBMC and on transformed B cells. However, since IgG⁺ B cells also express the p145 antigen, its expression cannot be directly linked to that of the μ -chain.

Two human B-cell markers, CD21 and CD22, with MW in the range of the bovine B-cell antigen reported here, have been described (Nadler, 1985; Ling *et al.*, 1987). Human CD21 has been shown to be the receptor for C3d (CR2) (Iida, Nadler & Nussenzweig, 1983) and for Epstein-Barr Virus (Shaw, Nemerow & Cooper, 1986). The bovine p145 antigen shares more characteristics with the human CD21 than human CD22. Both antigens are weakly expressed on peripheral blood B cells (compared to class II or Ig), but are more highly expressed on B cells isolated from lymph nodes (Nadler, 1985). The CD22 antigen is a dimer of p135 and p140 chains (Moldenhauer *et al.*,

1987; Ravoet *et al.*, 1987), whereas the precipitated bovine antigen always produced a single band, like human CD21. The MW of the bovine antigen increased after reduction (to 145,000), as reported for CD21 (Iida *et al.*, 1983), while no such change was reported for CD22 (Moldenhauer *et al.*, 1985). Also, in the histochemical analyses, the p145 antigen resembled CD21: antibodies to both antigens bind to follicular dendritic cells.

Despite all these similarities, only functional data or hybridization tests with genetic probes can unequivocally prove the homology between human CD21 and the bovine p145 B-cell antigen.

The mAb described here will be useful reagents for studying B cells using a marker other than Ig.

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