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Rapid cytotoxicity of human B lymphocytes induced by VH4-34 (VH4.21) gene-encoded monoclonal antibodies, II

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SUMMARY

We have previously described complement-independent killing of human B lymphocytes by two IgM MoAbs derived from the VH4-34 (VH4.21) gene. Analysis of 17 independently derived VH4-34-encoded MoAbs shows that B cell toxicity is not limited to the two described MoAbs, but is a general property shared by a subset of MoAbs derived from the VH4-34 gene. As observed by two independent microscopy techniques, giant membrane pores were formed on target B cells within 10–15 min of exposure to cytotoxic VH4-34-derived MoAbs. Toxicity by individual MoAb correlated directly to its B cell binding intensity measured by FACS, i.e. stronger the binding greater the killing. Sequence analysis showed that V_H region in germ-line or in near germ-line configuration was necessary but not sufficient for B cell binding. In addition, a particular sequence motif enriched in basic amino acids in the CDR3 may be required to supplement the reactivity mediated by the V_H region of the MoAb molecule. VH4-34-encoded antibodies that fulfil the above sequence requirements have cold agglutinin activity towards the i antigen of cord erythrocytes. *In vivo*, such anti-i/anti-B cell antibodies are rarely detected in healthy adults, but serum levels are dramatically elevated in selective pathological conditions, such as systemic lupus erythematosus and infectious mononucleosis. This strict regulation may be related to the novel and rapid mechanism of human B cell toxicity demonstrated by antibodies encoded by a single human V_H gene.

Keywords human B lymphocytes cytotoxicity i antigen cold agglutinins and membrane pores

INTRODUCTION

Cold agglutinins (CAs) are human autoantibodies, generally of the IgM class, which agglutinate erythrocytes at low temperature [1,2]. CAs have been classified as anti-i or anti-I depending on their ability to agglutinate cord (fetal and newborn) or adult erythrocytes, respectively. The I/i antigens are sugar moieties found on proteins and/or lipids of the erythrocyte membrane. Both antigens consist of repeating N-acetyllactosmine units, with i antigen being linear in structure and I antigen, branched. The expression of the I and i antigen is developmentally regulated [3]. The transition from i to I antigen occurs shortly after birth, with the acquisition of the branching enzyme, N-acetylglucaminyl transferase.

More than 20 years ago Williams *et al.* reported that the vast majority of antibodies with CA activity reacted with a polyclonal anti-serum generated against an isolated CA molecule [4]. The commonalty among all CAs was further characterized by a rat MoAb, 9G4, which recognizes the cross-reactive idiotope expressed on almost all human CAs with anti-I/i activity, but not with a panel of other human antibodies [5]. Subsequently, it was shown that the idiotope arises from the amino acid sequence of the VH4-34 gene-encoded heavy chain variable region [6]. Currently, apart from one anti-I antibody encoded by a V_H3 family gene [7], all other anti-I/i CAs ($n \geq 50$) are found to be derived from the VH4-34 gene [8–13]. Molecular analysis indicates that the idiotope recognized by MoAb 9G4 is conformation-restricted and dependent on a unique sequence near amino acids 23–25 in the framework 1 region of the variable heavy chain encoded by the VH4-34 gene [14]. Since these antibodies express different light chains and CDR3s, the I/i activity appears to be mediated by the immunoglobulin heavy chain encoded by the VH4-34 gene.

We have previously described two human anti-i CA MoAbs 216 and A6(H4C5), encoded by the VH4-34 gene that specifically bind and kill B lymphocytes [15]. Cytotoxicity was specific for $CD20^+$ mononuclear cells in human spleen and peripheral blood. The MoAbs were also cytotoxic to human B cell lines Nalm-6, Arent and OCI-LY8, but not to T cell lines PEER and HuT-78. The novel rapid toxicity was dependent on MoAb concentration and temperature of exposure, but was not dependent on complement

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activity. Scanning electron microscopy (SEM) demonstrated large pore-like structures in the membrane of human B cells treated with MoAbs 216 and A6(H4C5) [15]. The antigen recognized on B cells was sensitive to endo-β-galactosidase (an enzyme that cleaves the β1–4 linkage within N-acetyllactosamine units), suggesting it is a carbohydrate antigen similar in structure to the i antigen on cord erythrocytes [11,16]. 216 and A6(H4C5) were independently derived human anti-i MoAbs and have distinct CDR3s, J_H and light chains, implying that similar to the I/i specificity, binding and toxicity to B cells could also be regulated by the V_H region of the antibody molecule.

In this study we have extended our analysis of B cell binding and toxicity to 17 independently generated VH4-34 gene-encoded MoAbs. Our results confirm that the carbohydrate ligand on human B cells may indeed be similar in structure to the i antigen of cord erythrocytes, since all VH4-34-derived MoAbs that bound human B lymphocytes were of the anti-i specificity. Furthermore, toxicity of all MoAbs was significantly reduced following treatment of B lymphocytes with endo-β-galactosidase, suggesting that high-affinity binding of the carbohydrate ligand was essential for killing. Thus, we describe an autocrine form of human B cell death mediated by antibodies derived from a single human V_H gene. The rapid cytotoxic activity, independent of complement, independent of physiologic cellular processes and associated with membrane perturbations, has not been previously described.

MATERIALS AND METHODS

Human MoAbs

The human IgM MoAbs A6(H4C5) and 216 were prepared, processed and sequenced as previously described [16–18]. Production and purification of the human IgM MoAbs used as isotype-matched controls (MS2B6, B314/3, B314/9, and 91D5) has been described [19–21]. Production, processing and sequencing of the VH4-34-derived IgM MoAbs S20A2 [22], RT-2B [23], WW and HT [24], FS 2, FS 3, FS 4, FS 5, and FS 6 [10], Cal-4G, FS 9, and FS 11 [25] has been described. VH4-34-derived IgM MoAb H6-3C4 was a kind gift by Dr Shinzo Isojima (Advanced Fertility Centre, Fuchu Hospital, Japan) and was produced and processed as described [26]. Gee and FS 12 were produced in the laboratory of Dr F. Stevenson. Each MoAb was derived from a different patient. MoAbs 216, HT and WW came from patients with diffuse large cell lymphoma. MoAbs beginning with 'FS' were from patients with cold agglutinin disease. MoAbs RT2B, Cal-4G and S20A2 were from patients with infectious mononucleosis. MoAb A6(H4C5) was from a patient with Hodgkin's disease immunized with J5 lipopolysaccharide. MoAb Gee was from a patient with systemic lupus erythematosus and H6-3C4 was from a patient with infertility and anti-sperm antibodies. Antibody concentration was determined by human anti-IgM ELISA. All MoAbs were used as partially purified supernatants. Rat MoAb 9G4 was prepared as previously described [5] or purchased from Zymed Labs Inc. (South San Francisco, CA).

Cells and cell lines

Human adult spleen cells were obtained from patients who underwent splenectomy. Of the four spleen specimens, one was obtained from a trauma patient, two were removed during surgery for adrenal adenoma or pseudomyxenous cyst, and one was from a patient with spherocytosis. Heparinized adult peripheral blood ($n = 5$) was obtained from normal donors 20–50 years of age. All specimens were obtained with the approval of the Committee for the Protection of Human Subjects at Stanford University. Spleens were gently teased apart in Hanks' balanced salt solution (HBSS) with 1% fetal calf serum (FCS) and 0·2% DNase and passed through sterile nylon membranes to obtain single-cell suspensions. Mononuclear cells (MNC) from spleen and peripheral blood were separated by centrifugation through a Ficoll–Hypaque gradient (Histopaque 1077; Sigma, St Louis, MO) and were washed three times in PBS. Spleen MNC were frozen under liquid nitrogen in HBSS, containing 20% DMSO and 50% FCS, until use. Peripheral blood mononuclear cells (PBMC) were used fresh. Human B cell lines Nalm-6 [27] and OCI-LY8 [28] were maintained in logarithmic growth phase and seeded the previous day at 1×10^5 cells/ml in Iscove's medium with 10% FCS. The FCS was treated at 56°C for 1 h to inactivate complement.

Detection of B cell binding by FACS

Human spleen MNC, PBMC or B cell lines at $0{\cdot}5–1 \times 10^6$ cells/ml were incubated with VH4-34-encoded MoAbs or isotype-matched control human MoAbs (1·0 μg/ml) at 4°C in staining medium (PBS with 3% FCS and 0·01% Na azide) for 15 min. B lymphocytes were detected with PE-conjugated anti-CD20 (Becton Dickinson Immunocytometry Systems, San Jose, CA). Cells were washed twice in staining medium and incubated with anti-idiotypic antibody 9G4 (1·0 μg/ml, rat IgG2k) for 15 min, washed again and incubated with FITC-conjugated anti-rat κ antibody (Immunotech Inc., Westbrook, ME) for another 15 min. After washing, cells were resuspended in 200 μl PBS containing 3% FCS and PI (10 μg/ml; Sigma), and analysed on a modified dual-laser FACS II or a FACStar^{PLUS} (Becton Dickinson, Mountain View, CA) interfaced with a Vax 6300 computer (Digital Equipment, Maynard, MA) running FACS/Desk software [29]. In order to ensure reproducibility the sorter was calibrated with standard polystyrene micro spheres (Pandex, Mundelein, IL). Data were collected on 30 000 cells. PI-positive cells were excluded while measuring the mean channel fluorescence for each MoAb.

Cytotoxicity assay by FACS

Human spleen MNC or B and T cell lines at 1×10^6 cells/ml were exposed to VH4-34-derived MoAbs or isotype-matched control human MoAbs (7 μg/ml) at 4°C in staining medium for 0·5–2 h. B lymphocytes in MNC from spleen were detected using FITC-conjugated anti-CD20 (Becton Dickinson). The cells were then washed, resuspended in 200 μl PBS with 3% FCS and PI (10 μg/ml; Sigma), and analysed on FACS as described above. Dead and viable cells in each sample were calculated by appropriate PI gatings.

Scanning electron microscopy

Human spleen MNC (55–60% B lymphocytes) and Nalm-6 cells in staining medium were incubated with VH4-34-encoded MoAbs, control MoAb MS2B6 (5 μg/ml) or medium alone for 15 min at 4°C, washed three times with PBS and then fixed with 2% glutaraldehyde in 0·1 M sodium phosphate buffer pH 7·3 at 4°C for 2 h. The fixed cells were washed twice with 0·1 M sodium phosphate buffer, post-fixed for 1 h in 1% OsO_4, washed three times with distilled water, dehydrated in 30–100% ethyl alcohol and dried in hexamethyldisilazane [30]. Cells were mounted onto a double sticky Pelco carbon conductive stub. The stub was gold-coated on a Polaron 5300 for 5 min, and the cells were examined by a Philips 505 Scanning Electron Microscope (Philips, Einhoven, Holland).

Confocal microscopy

Nalm-6 cells in staining medium were incubated for 15 min at 4°C with biotin-conjugated 216 or MS2B6 (5 μg/ml), washed three

Table 1. VH4-34 gene-derived MoAbs with anti-i activity bind and kill human B lymphocytes

Human IgM MoAb	Erythrocyte specificity	Light chain	Mean channel fluorescence*				Percent viable cells†		
			Nalm-6	OCI-LY8	Spleen B cells‡	Hu PBL B cells	Nalm-6	OCI-LY8	Spleen B cells‡
216§	i	λ	190	182	200	237	4	18	12
RT-2B§	i	λ	180	177	182	232	10	50	32
FS 12	i	κ	174	162	NT¶	NT	18	55	28
A6(H4C5)§	i	κ	166	162	188	230	32	67	35
Cal-4G§	i	λ	153	144	168	222	38	68	40
S20A2	i	λ	152	NT	160	186	41	NT	49
FS 3	i	λ	139	165	144	180	40	65	56
Gee	i	κ	98	140	116	164	55	82	62
HT	i	λ	71	112	106	139	70	91	80
FS 5	i	κ	55	82	86	142	84	96	86
H6-3C4	i	λ	55	65	79	128	95	94	95
WW	I	κ	40	38	NT	NT	93	90	94
FS 2	I	κ	41	39	NT	NT	94	93	NT
FS 9	I	κ	27	32	NT	NT	94	91	NT
FS 11	I	κ	25	28	44	39	96	95	94
FS 4	I	κ	17	16	52	49	94	97	96
FS 6	I	κ	17	20	48	46	96	95	96
Controls									
MS2B6**		λ	18	20	34	36	96	94	94
9G4/anti-k		NA¶	17	18	38	35	NA	NA	NA
anti-k		NA	18	20	28	28	NA	NA	NA
Cells only		NA	16	19	30	30	96	97	95

* Fluorescence detected by FACS on cells using unlabelled VH4-34 MoAbs (1·0 μm/ml) followed by anti-idiotope 9G4 (1·0 μg/ml, rat IgG/k) and detected with FITC-conjugated mouse anti-rat κ. Data represent a single experiment. All MoAbs were assayed on each cell type at least three times, giving comparable results.

† Cells were incubated at 4°C for 1 h with MoAb concentrations of 7 μg/ml. Secondary antibodies were not used in the cytotoxicity assay. Viable cells were determined by exclusion of PI by FACS. Data represent a single experiment. All MoAbs were assayed on each cell type at least three times, giving comparable results. Cytoxicity by MoAbs decreased by at least three-fold when tested on target B cells treated with enzyme endo-β-galactosidase.

‡ B cells were determined by staining with PE-conjugated anti-CD20 and gating on $CD20^+$ cells. No cell death was seen in the $CD20^-$ population (T cells, monocytes and natural killer (NK) cells). Spleen cells used for staining and cytotoxicity were from different individuals.

§ Four MoAbs were tested for pore formation by scanning electron microscopy (SEM) (see Figs 2 and 3). Other MoAbs in the panel were not tested for pore formation.

¶ NT, Not tested; NA, not applicable.

** Other isotype-matched control human MoAbs ($n = 8$) gave background fluorescence and ≥95% viability.

times with staining medium, incubated with avidin-conjugated Texas red (Becton Dickinson), washed again and resuspended in staining medium for microscopy. Imaging was performed at the Cell Sciences Imaging Facility on the Multi Probe 2010 laser confocal microscope (Molecular Dynamics, Sunnyvale, CA). The Multi Probe uses an Ar/Kr mixed gas laser with excitation lines of 488, 568 and 647 and is built on a Nikon Diaphot inverted microscope. An excitation wavelength of 488 nm was selected, the emitted light was passed through a 510 LP beamsplitter and collected with a 510 long pass filter. A Nikon×60 (NA 1.4) planapo objective was used. Images of serial optical sections were recorded every 1·0 μm per vertical step along the *Z*-axis. Each *Z*-series was compiled together to generate a maximum intensity projection image and merged with Adobe Photoshop v 3.0.

RESULTS

VH4-34-gene derived MoAbs with anti-i activity, but not anti-I activity, bind and kill human B lymphocytes

Of the 17 IgM MoAbs encoded by the VH4-34 gene in our panel, 11 were specific for the i antigen and six for the I antigen of cord and adult erythrocytes, respectively. All 17 MoAbs (anti-I and anti-i) bound the anti-idiotypic antibody, 9G4 (rat IgGk) with similar relative solid-phase affinities [31]. For B cell binding and toxicity, human spleen MNC, PBMC, B cell lines Nalm-6 and OCI-LY8 were treated with individual MoAbs and analysed on FACS as described in Materials and Methods. Table 1 ranks the VH4-34-encoded MoAbs in decreasing order of intensity for B cell binding. The VH4-34-encoded MoAbs span a spectrum, ranging from strong binders to non-B cell binders compared with the isotype-matched control MoAb MS2B6 (derived from a member of the V_H3 gene family). Other control human IgM MoAbs ($n = 8$), encoded by V_H1, V_H3, V_H4 (other than VH4-34) and V_H5 gene families, gave fluorescence values comparable to MoAb MS2B6 (data not shown). A striking correlation emerges when B cell binding is compared with the erythrocyte specificity of each MoAb. All anti-i MoAbs bound human B cells, albeit with varying intensity. At a given MoAb concentration, anti-i MoAb 216 showed the greatest B cell binding and H6-3C4 the weakest. Cytotoxicity was directly proportional to the binding intensity and

	FR1	CDR1	FR2	CDR2
	1		36	50
VH4-34 (GL):	QVQLQQWGAGLLKPSETLSLTCAVYGGS	FSGYYWS	WIRQPPGKGLEWIG	EINHSGSTNYNPSLKS
216*:	----------	-------	----------	----------
RT2B*:	----------	-------	----------	----------
FS 12:	----------	------T	----------	----------
A6H4C5*:	----------	-------	----------	----------
Cal4G* :	----------	-------	----------	----------
S20A2*:	----------	-------	----------	----------
FS 3:	----------	-------	----------	---Y----------
Gee*:	----------	-------	----------	----------
HT*:	----------	-------	----------	----------
FS 5*:	----------	-------	----------	----------
H6-3C4:	----------D-----	-------	----------	----------R-
WW:	----------	-------	----------	---NYEN---------
FS 2:	--H-----T----------	--D----	----------	---D--I---------
FS 9:	----H----------E-	-------	----------	-V--D----------
FS 11:	----------	---y--I	----------	---------C------
FS 4:	----------P---------	--D---T	----------	----------
FS 6:	----H----------	------T	----------	----------L----

	FR3	CDR3	JH	Net CDR3 charge
	66			
VH4-34:	RVTISVDTSKNQFSLKLSSVTAADTAVYYCAR			
216*:	----------	G**R**MV**R**GA**K**YN WFDP	WGQGTLVTVSS[JH5]	+2
RT2B*:	----------	G**RR**QYQLTQ YFQH	WGQGTLVTVSS[JH1]	+2
FS 12:	-------K----------	G**R**SS**E**R**L**SPP**R** YFDL	WGRGTLVTVSS[JH2]	+1
A6H4C5*:	----------	**K**AP**RR**LGL YYYYGMDV	WGQGTTVTVSS[JH6]	+2
Cal4G*:	----------	**R**IMYCSGGSCYSAGW**R** Y	WGQGTLVTVSS[JH4]	+2
S20A2*	----------	G**R**FYVVPAAMHGTLFCAF YYMDV	WGQGTLVTVSS[JH6]	0
FS 3:	----------	GT**R**SWYPSD FDY	WGQGTLVTVSS[JH4]	-1
Gee*:	----------	G**R**EGGNS**K**Y YFDY	WGQGTLVTVSS[JH4b]	0
HT*:	----------	GS**K**IAA**R**PPDG Y	WGQGTLVTVSS[JH4]	+1
FS 5*:	----------	GPYYIDDSSGYYPG ...	WGQGTLVTVSS[JH4]	-2
H6-3C4:	----------R----------	GFMV**R**GIMWN YYYMDV	WGKGTTVTVSP[JH6]	0
WW:	--I----------	VFYYDSSG YPDY	WSQGTLVTVSS	-2
FS 2:	----------L-----	EWGSGAYYPYY FDY	WGQGNLVTVSS[JH4]	-2
FS 9:	--N----------T------A-------	GPALMITG FQY	WQQGTLVTVSS[JH4b]	0
FS 11:	---M----sM-----K-T----------	GDTVVVPS**R**P DY	WGQGTLVTVSS[JH4a]	-1
FS 4:	-D----------NVN-L----------	**R**LGSGSYPN FDY	WGQGNLVTVSS[JH4]	0
FS 6:	----------Q----------	ALGDGSTEGLP DY	WGRGTLVTVSS[JH4]	-3

Fig. 1. VH4-34 MoAbs with anti-i specificity have fewer substitutions in the V_H region compared with VH4-34 anti-I MoAbs. The germ-line sequence of the VH4-34 gene is shown on top. MoAbs are listed in the same order as in Table 1 (i.e. in descending order for B cell binding and cytotoxicity). The first 11 MoAbs have anti-i specificity and the last six have anti-I specificity. *MoAbs which encode the VH4-34 gene in germ-line configuration. VH4-34-derived MoAbs with anti-i/anti-B cell specificity have increased numbers of basic amino acids in their CDR3. Amino acid sequence of CDR3 and J_H region, the net charge of CDR3 and the type of light chain used by the 17 VH4-34-encoded MoAbs are shown. Amino acids with basic side chains are shown in bold and the amino acids with acidic side chains are underlined. To obtain the net charge in the CDR3, amino acids were given a value of −1 (negative), 0 (neutral) or +1 (positive) depending on their respective side chains. The sum gives the final net charge for this region. Accordingly, MoAb 216 has three amino acids with positively charged side chains and one amino acid with a negatively charged side chain, hence the net charge of the CDR3 region of MoAb 216 is +2. All MoAbs are encoded by independent D and J_H genes and express independently derived light chains.

appeared to be independent of the light chain isotype, since MoAb 216 is a λ and MoAb FS 12 is *κ*, but both were strong binders and killers. Compared with anti-i MoAbs, six anti-I MoAbs tested bound B cells very weakly and demonstrated no cytotoxicity (Table 1). In fact, the B cell binding by anti-I MoAbs was comparable to some of the low binding, almost non-toxic anti-i MoAbs, FS 5 and H6-3C4.

Sequence analysis of VH4-34 gene-derived MoAbs

The observation that six anti-I MoAbs derived from the VH4-34 gene do not bind B lymphocytes suggests that the variable heavy chain region appears to be necessary but is not sufficient for B cell binding. Examination of the protein sequence reveals that all of the anti-I MoAbs have a number of substitutions in the variable heavy chain region (Fig. 1). These amino acid substitutions are scattered

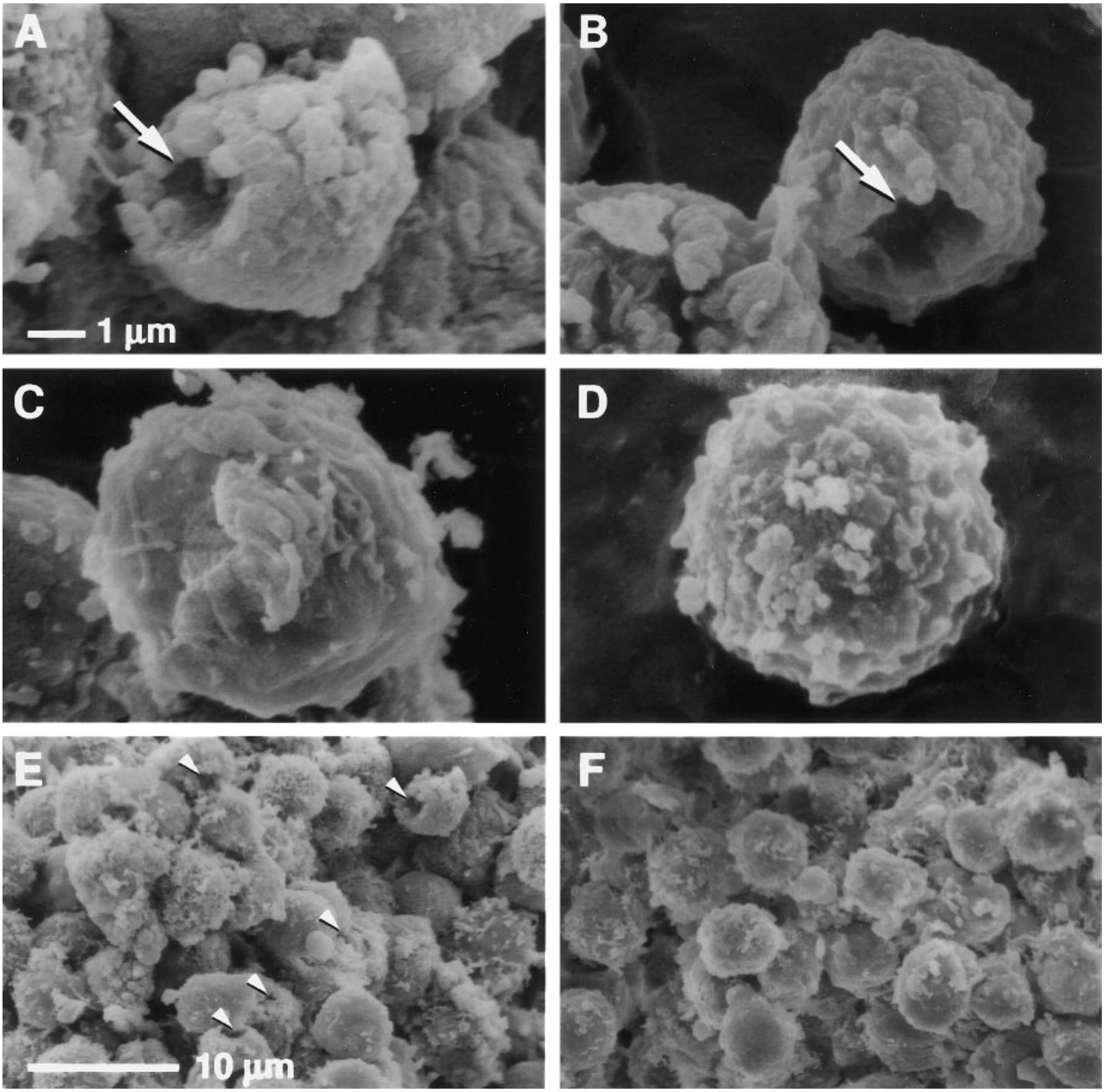

Fig. 2. Human spleen B lymphocytes exposed to MoAbs 216 and A6(H4C5) develop membrane pores. Spleen mononuclear cells (MNC) were incubated with 5 μg/ml of MoAbs 216 (A), A6(H4C5) (B), control MoAb MS2B6 (C) or kept in medium alone (D) for 15 min at 4°C and then processed for scanning electron microscopy (SEM) mounting. The arrow demonstrates the position of the membrane pores. The magnifications of A–D are the same. Overviews of cells treated with MoAbs 216 (E) and MS2B6 (F) show that multiple cells develop pores when exposed to MoAb 216 (arrowheads). The magnifications of E and F are the same.

throughout the framework and hypervariable regions. In contrast, eight of the 11 anti-i MoAbs are derived from the VH4-34 gene in a somatically unmutated configuration, and three have limited substitutions.

A second weak correlation appears when B cell killing ability is compared with the amino acid sequence in the CDR3 of each MoAb molecule. All strong binders have a predominance of basic amino acids, arginine (R) or lysine (K) in their CDR3 (Fig. 1). In general, compared with anti-I MoAbs, MoAbs with anti-i specificity have a higher net positive charge in their CDR3, and among anti-i MoAbs, those with a higher net positive charge, 216, FS 12, RT-2B and A6(H4C5), were more cytotoxic than other anti-i MoAbs FS 5, HT and FS 3 (Fig. 1). Thus, cytotoxicity may correlate directly with the net charge carried by the CDR3 for each MoAb (i.e. the greater the net positive charge in the CDR3, the more the toxicity), or alternatively be dependent upon the presence of a permissive sequence motif enriched in basic amino acids in the CDR3.

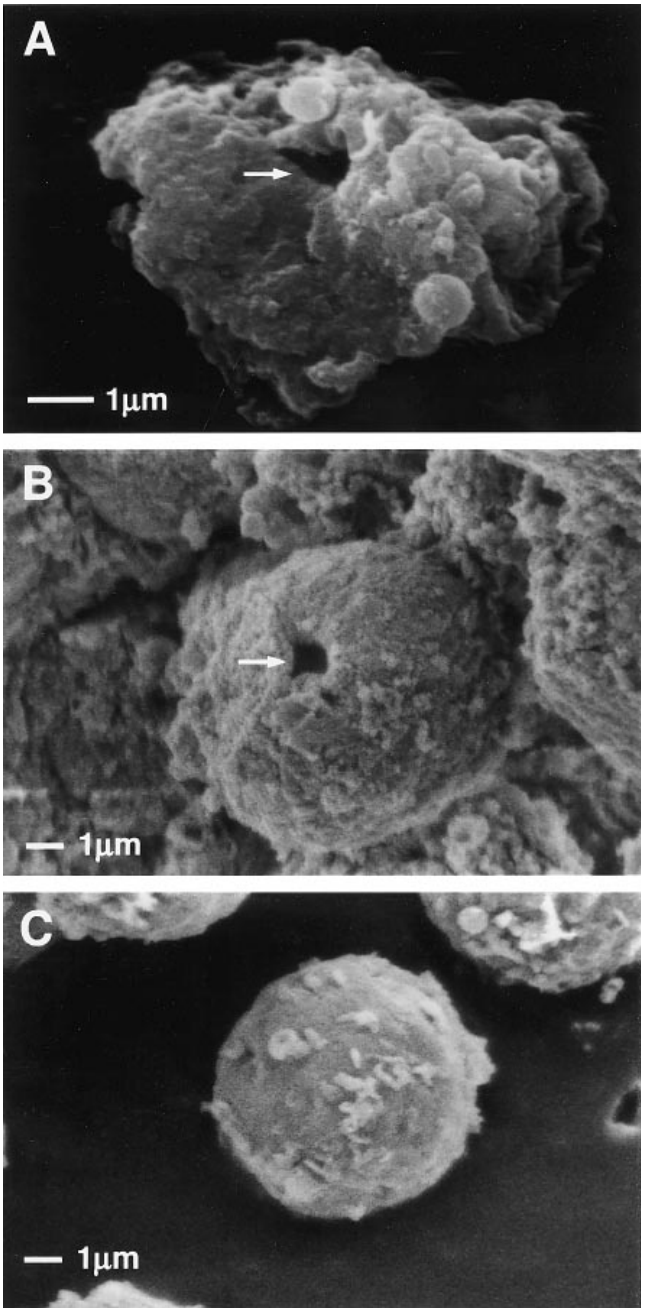

Fig. 3. VH4-34-derived MoAb-treated Nalm-6 cells (a human pre-B cell line) develop membrane pores. Cells were incubated with MoAbs RT-2B (7 μg/ml, A), Cal4G (10 μg/ml, B), MS2B6 (12 μg/ml, C) for 15 min at 4°C and then processed for scanning electron microscopy (SEM) mounting. The bar in each frame corresponds to 1 μm. The arrow demonstrates the position of the membrane pores.

B cells treated with VH4-34-encoded MoAbs develop membrane pores as detected by SEM and confocal microscopy

We have previously reported formation of membrane pores on B cell line Nalm-6 exposed to MoAbs 216 and A6(H4C5) by SEM [15]. Here we show that normal B cells from human spleen also develop similar membrane holes following exposure to MoAbs 216 and A6(H4C5) (Fig. 2). Spleen MNC exposed to control MoAb or maintained in medium alone do not exhibit membrane

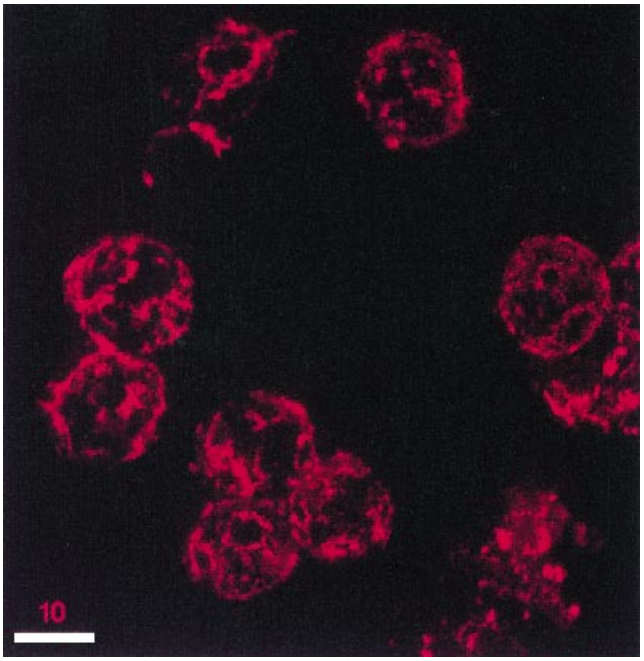

Fig. 4. Membrane pore formation detected by confocal microscopy on Nalm-6 cells following exposure to VH4-34-encoded MoAb 216. Individual frames of a Z-series (cross-sectional images taken at 1-μm intervals from the top to the base of the cells) were compiled together to generate the above maximum intensity projection image. The bar corresponds to 10 μm. Cells treated with control MoAb MS2B6 were completely devoid of fluorescence.

pores. The overview (Fig. 2E) shows that cells exposed to VH4-34-encoded MoAbs appear to be damaged, with increased blebbing at the cell surface, compared with cells exposed to control MoAb (Fig. 2F). Generally a single pore was seen on individual cells. Although cells with multiple pores were seen, such cells were in a state of disintegration and had almost lost their cellular identity. Formation of a single pore could lead to osmotic swelling and degeneration, making it difficult to find an intact cell with a second pore.

Furthermore, such a dramatic change in cell morphology was not restricted to MoAbs 216 and A6(H4C5). Two other anti-i MoAbs tested (RT-2B and Cal4G) also caused such membrane perturbations in Nalm-6 cells (Fig. 3). Thus, the morphology of the pore appears to be similar regardless of the target cell type (normal human spleen B or transformed human B cell line) and cytotoxic anti-i VH4-34-encoded MoAb combination, suggesting it is a general mechanism of B cell cytotoxicity used by this set of human antibodies.

Since the antigen recognized on human B cells is structurally similar to the i antigen on cord erythrocytes, we analysed the effect of VH4-34 gene-derived MoAbs on cord erythrocytes with respect to cell death/lysis and pore formation. To detect pore formation, cord erythrocytes (5×10^6) were incubated with MoAb 216 (50 μg/ml) at 4°C for 20 min and then processed for SEM mounting similar to Nalm-6 cells. For the detection of lysis, cord erythrocytes (5×10^6) were incubated with MoAbs 216 and A6(H4C5) (50 μg/ml) at either 4°C or 37°C for 20 min to 4 h, and then visually examined for haemolysis. No erythrocyte lysis or pore formation was demonstrated following incubation of cord erythrocytes with

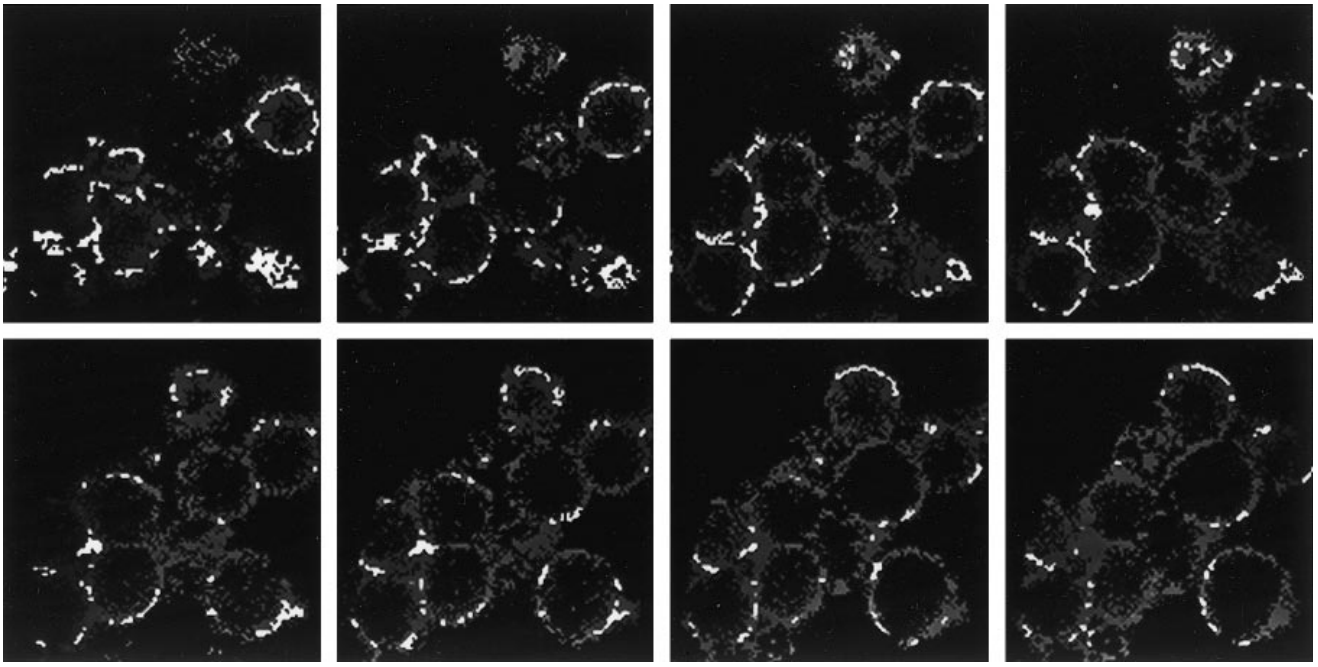

Fig. 5. MoAb 216 is not internalized by Nalm-6 cells. Cross-sectional images were taken at 1-μm intervals from the top to the base of cells in the field. Representative images of eight confocal planes are shown, with distance intervals of 2 μm. The base of the cells in the field is not shown. Magnification of all panels is $\times$ 600.

MoAbs 216 and A6(H4C5). The contrast in the response of cord erythrocytes and B lymphocytes could be a reflection of membrane and cytoskeletal differences between the two cell types.

Confocal microscopy (CFM) was used to study the cell morphology and to track the site of MoAb binding in the context of the membrane pore. Nalm-6 cells were exposed to biotin-conjugated MoAb 216 (5 μg/ml) for 15 min at 4°C, followed by Texas red-conjugated avidin and observed under a confocal microscope as described. Figure 4 shows formation of rings or circles that are devoid of fluorescence on several Nalm-6 cells. These rings appear to be morphologically similar to the pore demonstrated by SEM [15]. Several cells show formation of multiple rings and appear to be physically intact, in contrast to the impairment accompanying cells with multiple pores under SEM. Analysis of cross-sectional images shows that 216 reactivity remains along the cell surface and is not internalized following binding (Fig. 5). The plasma membrane defect demonstrated by both SEM and CFM is significantly larger than the pores formed by other well known pore-forming proteins, such as C9 complement component and perforin, and appears to be a novel mechanism of cell death mediated by VH4-34 gene-encoded antibodies in a complement-independent manner.

DISCUSSION

Several previous studies with CAs have shown that most anti-i antibodies are derived from the VH4-34 gene in germ-line configuration [8,10]. In this study we report that such antibodies are cytotoxic to human B lymphocytes. Although the carbohydrate ligand on B cells and cord erythrocytes appears to be similar, the effect of MoAb binding on the two cell types is very different. Following *in vitro* exposure to VH4-34 gene-derived MoAbs, human B lymphocytes are killed in a complement-independent mechanism, whereas cord erythrocytes are agglutinated. Differences in cytoskeletal structure may explain the varied response of the two cell types. The cytoskeleton of the erythrocyte is attached to the plasma membrane at several points to form a shell-like structure that gives its membrane strength and flexibility. In contrast, the cytoskeleton of most nucleated cells courses through the cytoplasm and is anchored to the membrane only at a few positions [32]. Alternatively, since not all anti-i MoAbs are cytotoxic (MoAbs FS 5 and H6-3C4), the ligand on B lymphocytes may be functionally and physically distinct from the i antigen on cord erythrocytes. A pan leucocyte antigen, CD45, which is not present on the membranes of erythrocytes and which expresses the N-acetyllactosamine side chain, has been suggested to be the carrier for the i-like specificity on human B lymphocytes [13,33].

Exposure of B cells to VH4-34-encoded MoAbs is accompanied by formation of an unusual giant pore-like structure in the cell membranes, as demonstrated by two techniques in microscopy. Recently Matsuoka *et al.* have described a rat MoAb 'RE2' that also forms similar membrane pores [34]. The pores formed by RE2 and VH4-34-derived MoAbs are giant (> 600 nm) in comparison to the pores formed by complement and perforin (range 5–35 nm) [35]. Although single pores are common, we have observed cells with multiple pores on both SEM and CFM. However, under SEM cells with multiple pores had totally lost their membrane integrity, almost resembling dead cells. The extensive processing required for SEM mounting could destroy an already fragile cell, compared with the direct observation of cells under CFM. The rapid antibody action, independent of active metabolism and accompanied by large membrane deformations, suggests that the mechanism leading to death is a biophysical phenomenon involving the cellular cytoskeleton, and resembles necrosis rather than the energy-dependent process of apoptosis.

VH4-34 gene-derived antibodies have been traditionally described as autoantibodies. Besides the I/i antigen on erythrocytes, VH4-34 gene has been reported to encode antibodies against auto-, allo- and exogenous antigens, such as DNA, cardiolipin, Rh, and lipid-A, respectively [36]. Our panel of VH4-34-derived MoAbs do react with some of the above mentioned antigens. MoAbs 216, A6(H4C5), Cal-4G and HT react with ssDNA and cardiolipin, whereas only 216 and A6(H4C5) bind lipid-A [16]. Since no obvious sequence differences are apparent in the V_H region of the MoAb, relative contributions from the V_L chain and the CDR3 may explain the differential reactivity to these exo/autoantigens.

Our study suggests that presence of basic amino acids in the CDR3 may enhance or contribute to the B cell reactivity encoded by the VH4-34 gene in germ-line configuration. In support of this hypothesis, a recent study by Li *et al.* demonstrated that the CDR3 influenced the fine specificity of I antigen binding by VH4-34 gene-encoded antibodies [37]. In contrast, a study by Parr *et al.* suggested that B cell binding by VH4-34-encoded IgMs was not dependent on the CDR3 [13]. In that study, the CDR3 of a VH4-59-encoded MoAb, which did not have B cell binding activity, was genetically recombined with a VH4-34 gene, derived from an antibody with B cell binding activity. The chimaeric antibody was found to bind B cells as well as or even better than the parent MoAb. However, sequence examination of this chimaeric antibody revealed the presence of basic amino acids in the CDR3. If our hypothesis is correct, the protein derived from this genetic construct would be capable of binding B lymphocytes. A similar study with a CDR3 with a net zero or negative charge might confirm the influence of CDR3 in B cell binding.

In vivo, the expression of VH4-34 gene-derived antibodies is strictly regulated. As detected by anti-idiotypic antibody 9G4, serum levels of VH4-34-derived antibodies are negligible or low in normal adults [38]. Increase in circulating VH4-34-derived antibodies is seen only in selective pathological conditions associated with B cell dysfunction. Among autoimmune diseases, an increase in VH4-34-derived antibodies is seen in systemic lupus erythematosus (SLE) and juvenile chronic arthritis, but not in rheumatoid arthritis, myositis and scleroderma [39–41]. In B cell malignancies, tumour cells preferentially express the VH4-34 gene product only in diffuse large cell lymphoma [24,42,43]. Among bacterial and viral infections, serum levels of VH4-34-derived antibodies are dramatically elevated by *Mycoplasma pneumoniae* (anti-I specificity), HIV and Epstein–Barr virus (EBV) (anti-i specificity), but not by *Klebsiella pneumoniae* or *Mycobacterium tuberculosis* [2,44,45].

The function of VH4-34-derived antibodies in disease development and/or regulation remains unclear. Over-representation only in selective conditions suggests a specific role in host defence against certain antigens and/or pathogens. However, sequence analysis of antibodies rescued from SLE patients demonstrates that VH4-34-derived immunoglobulins fulfilling the B cell cytotoxicity criteria are found circulating in patient's serum [40,46]. Furthermore, our preliminary analysis shows that VH4-34-derived IgM antibodies obtained from SLE patients are cytotoxic to human B cells as tested *in vitro*. This toxicity could influence immune regulation by curtailing the polyclonal B cell proliferation associated with the disease, or alternatively, the release of cellular DNA following B cell lysis may lead to the induction of the pathogenic anti-DNA response classically associated with SLE.

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