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

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SUMMARY

We have previously described two human cold agglutinin MoAbs 216 and A6(H4C5), that are derived from the VH4-34 (VH4.21) gene that bind specifically to a cell surface ligand on human B lymphocytes. In this study, we report that binding of 216 and A6(H4C5) leads to rapid killing of target B cells. This complement-independent cytotoxicity was measured by three independent assays, cell viability dye uptake on FACS, ^{3}H-thymidine uptake, and the 3(4,5)-dimethylthiazol-2,5-diphenyl tetrazolium bromide (MTT) assay. 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, OCI-LY8, Arent and SUP-B8, but not to T cell lines HuT 78 and PEER. As observed by scanning electron microscopy, membrane pores were formed within 15 min of exposure to the MoAbs. Cytotoxic activity was dependent on MoAb concentration and temperature of exposure. Killing was greater at 4°C than 37°C. Sodium azide and EDTA did not block the cytotoxic activity. No DNA fragmentation typical of apoptosis was observed. This rapid cytotoxic activity, independent of physiologic cellular processes and independent of complement, suggests a novel mechanism of cell death via membrane perturbations.

Keywords human B lymphocytes cytotoxicity monoclonal antibodies i antigen

INTRODUCTION

Cold agglutinins (CAs) are human autoantibodies, generally of the IgM class, which agglutinate erythrocytes at low temperature. The major CAs bind either the I or i carbohydrate antigen present on adult or cord erythrocytes, respectively. Both antigens consist of repeating N-acetyllactosamine units, with i antigen being linear in structure and I antigen branched [1,2]. Recent studies by several laboratories have demonstrated a restricted V_H gene usage among CAs. The heavy chain variable gene, VH4-34 (VH4.21) has been found to encode many anti-I/i CAs [3–6]. This strong restriction suggests that the VH4-34 gene product encodes the anti-I/i CA activity.

We have previously described two human CA MoAbs 216 and A6(H4C5), encoded by the VH4-34 gene, that bind specifically to human B lymphocytes [7]. The antigen recognized on B cells is sensitive to the endo-β-galactosidase (an enzyme that cleaves the β1–4 linkage within N-acetyllactosamine units), suggesting that it is a carbohydrate epitope similar in structure to the i antigen on cord erythrocytes [6–8]. 216 and A6(H4C5) are independently derived MoAbs with distinct CDR3s, J_H and light chains [7]. The two MoAbs share only the V_H region, which is germ-line in configuration, suggesting that similar to the I/i specificity, binding to B cells may also be regulated by the V_H region of the antibody. A recent study by Parr *et al.* showing that B cell binding by VH4-34-encoded IgM is not dependent on *in vivo* somatic selection in the CDR3 region is consistent with this finding [9].

Here we report that the binding of the two human MoAbs to B lymphocytes leads to rapid killing of the target cells. The cytotoxic effects of the two MoAbs are significantly reduced following treatment of target cells with endo-β-galactosidase, suggesting that specific surface binding of the carhohydrate ligand on B cells is essential for killing. The mechanism of cell death is distinct from apoptosis or complement-mediated necrosis, and appears to be similar to a novel form of cell death recently reported by Matsuoka *et al.* [10].

MATERIALS AND METHODS

Production and purification of human MoAbs

The human IgM MoAbs A6(H4C5) and 216 were prepared and purified in our laboratory as previously described [7,11,12]. Production and purification of the human IgM MoAbs used as isotype-matched controls (MS2B6, B314/3, B314/9, and 91D5) has been described earlier [13–15]. MoAb concentration was determined by ELISA and the MoAbs were biotinylated with

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N-hydroxysuccinamide-biotin as described [13]. All purified MoAbs were >90% pure IgM as assayed by PAGE.

Cells and cell lines

Human adult splenic cells were obtained from patients who underwent splenectomy. Of the six spleen specimens, four were obtained from trauma patients, one was removed during surgery for adrenal adenoma, and one was from a patient with spherocytosis. Heparinized adult peripheral blood specimens ($n = 5$) were 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. Splenocytes and peripheral blood specimens were centrifuged through a Ficoll–Hypaque gradient (Histopaque-1077; Sigma, St Louis, MO). The mononuclear cells were washed three times in PBS. All splenic mononuclear samples had been frozen in HBSS containing 20% DMSO, 50% FCS under liquid nitrogen. Peripheral blood mononuclear cells (PBMC) were used fresh.

Human B cell lines Nalm-6 [16], SUP-B8 [17], Arent [18] and OCI-LY8 [19], and T cell lines PEER [20] and HuT 78 [21] have been described. Human B cell lines SUP-B8 and OCI-LY8 were a kind gift from Dr Shoshana Levy (Stanford University). To obtain consistent results in the cytotoxicity assays, cell lines 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.

Propidium iodide uptake by FACS

Human splenic mononuclear cells, PBMC or B and T cell lines at $1–2 \times 10^6$ cells/ml were exposed to different concentrations of VH4-34 MoAbs or isotype-matched control human MoAbs, at 37°C or 4°C, in various media and time intervals as specified in each experiment. After incubation with the human MoAbs, splenic mononuclear and PBMC were stained using the manufacturer's protocol with FITC-conjugated anti-CD20 (Becton Dickinson Immunocytometry Systems, San Jose, CA) to label B lymphocytes. The cells were then washed, resuspended in 200 μl PBS with 3% FCS and propidium iodide (PI; 10 μg/ml; Sigma), and analysed on a highly modified dual-laser FACS II or a FACStar (Becton Dickinson, Mountain View, CA) interfaced with a Vax 6300 computer (Digital Equipment, Maynard, MA) running FACS/Desk software [22]. In order to ensure reproducibility, the sorter was calibrated with standard polystyrene micro spheres (Pandex). Data were collected on 30 000 cells.

Enzyme treatment of Nalm-6 cells

Nalm-6 cells were incubated overnight at 3×10^6 cells/ml in Iscove's with 10% FCS and 50 mU/ml of endo-β-galactosidase (Boehringer, Mannheim, Germany) at 37°C. Enzyme-treated and control samples were stained with PE-conjugated CD19, FITC-conjugated CD71 (Becton Dickinson) and biotinylated MoAb 216 (1 μg/ml) for 15 min on ice. The cells were washed and counter stained with Texas red-conjugated streptavidin (Becton Dickinson), washed again resuspended in staining medium with PI and analysed on a dual laser FACS II as described. For cytotoxicity, enzyme-treated and untreated Nalm-6 cells were exposed to MoAbs 216 and MS2B6 (10 μg/ml) and processed as described.

MTT assay

We used a modified version of the viable cell assay, using 3(4,5)-dimethylthiazol-2,5-diphenyl tetrazolium bromide (MTT) [23]. Pre-B cell line Nalm-6 at 2×10^5 cells/ml was suspended in Iscove's 10% FCS with VH4-34 or isotype-matched control MoAb (10 μg/ml), and transferred as quadruplicates to 96-well flat-bottomed plates (Nunc, Naperville, IL). Plates were incubated at 37°C for 18 h. MTT (Sigma) was added at a final concentration of 200 μg/ml, and the cells were incubated for 4 h. A purple formazan product is formed by the action of mitochondrial enzymes in living cells. The plates were spun, the supernatant removed and the intracellular purple product solubilized by the addition of DMSO. Absorbance was quantified at 540 nm in optical density (OD) units on an ELISA reader (Titertek Multiscan plus; ICN, Costa Mesa, CA). Background OD was subtracted.

Thymidine uptake assay

Cells were prepared as in the MTT assay, except dialysed FCS was used. ^{3}H-thymidine 1 μCi/well (Amersham Life Sciences, Arlington Heights, IL) was added to cells for the last 4 h. The cells were harvested on filter paper strips, washed, and counted in the scintillation counter.

Scanning electron microscopy

Nalm-6 cells in PBS with 3% FCS were incubated with 216, A6(H4C5), or 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 and dehydrated in 30–100% ethyl alcohol followed by drying in hexamethyldisilazane [24]. The 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 Philips 505 Scanning Electron Microscope (Philips, Eindhoven, Holland).

DNA fragmentation assay

Extraction and electrophoresis of DNA from human B cell lines exposed to VH4-34 gene-encoded MoAbs, isotype-matched control MoAbs or untreated cells was performed as described [25].

RESULTS

VH4-34-encoded MoAbs 216 and A6(H4C5) are cytotoxic for normal human splenic and peripheral blood B lymphocytes

While studying the surface staining of B lymphocytes by VH4-34 MoAbs we found that these samples had a significantly greater proportion of cells with FACS scatter signals typical of dead cells than cells treated with isotype-matched control antibody or medium alone. Similarly, the percentage of cells that stained with viability dye, PI, was significantly greater in cells treated with VH4-34 MoAbs than control cells. We used both scatter changes and PI uptake to compare the frequency of dead cells in VH4-34 MoAb-treated samples. By scatter signal analysis only 35–40% of splenic $CD20^+$ cells were viable in VH4-34 MoAb-treated samples compared with $\approx$ 80% viable cells in control samples (Fig. 1). By PI analysis, 25% of B cells treated with VH4-34 MoAbs were viable compared with > 75% in isotype-matched control and medium alone samples (Fig. 1). Splenic mononuclear cells treated with non-VH4-34 human IgM MoAbs

B314/3, B314/9 and 91D5 [15] did not bind human B cells, or show an increase in PI uptake or changes in scatter measurements (data not shown).

Cytotoxicity of 216 and A6(H4C5) was specific for peripheral blood B lymphocytes, as demonstrated in Fig. 2. After incubation with VH4-34 MoAb 216, peripheral blood $CD20^+$ cells were all stained with PI, whereas the $CD20^-$ cells (which includes T cells, monocytes and natural killer (NK) cells) were clearly PI-negative. In the samples incubated with control MoAb MS2B6 or medium alone, both $CD20^+$ and $CD20^-$ cells were all viable (PI^-) cells.

VH4-34-encoded MoAbs 216 and A6(H4C5) are cytototoxic to neoplastic and transformed human B cell lines

Binding studies with haematopoietic cell lines representative of different lineages and stages of B cell development confirm the B cell specificity of 216 and A6(H4C5). 216 and A6(H4C5) bound pre-B cell line Nalm-6 and mature B cell lines Arent, OCI-LY8 and SUP-B8 (Table 1). The cytotoxicity of the two MoAbs correlated directly to their binding intensity measured by FACS, i.e. the stronger the fluorescence the greater the killing ability. Percentage of viable cells (PI^-) after incubation with VH4-34 MoAbs and control MoAb is shown in Table 1. Treatment of Nalm-6 cells with endo-β-galactosidase significantly reduced both the binding and cytotoxic activity, suggesting that binding of the carbohydrate i-like antigen is essential for the cytotoxic effects of VH4-34 MoAbs. The MoAbs did not bind or kill T cell lines HuT 78 and PEER (Table 1). Isotype-matched human MoAbs MS2B6, B314/3, B314/9 and 91D5 did not bind or kill any of the T or B cell lines tested. Toxicity was directly dependent on MoAb concentration and was relatively rapid, since PI^+ cells were observed within 5 min of MoAb exposure (data not shown). Furthermore, PI uptake was not due to transient changes in the membrane, since sorted PI^+ cells returned to culture did not survive.

Cytotoxicity of 216 and A6(H4C5) was first observed while studying their B cell binding properties at 4°C. To study the effect of the two MoAbs at physiologic temperature, cytotoxicity was measured by ^{3}H-thymidine uptake and MTT assay, which measure DNA replication and the function of mitochondrial enzymes, respectively. Both VH4-34 MoAbs 216 and A6(H4C5) inhibited thymidine uptake and mitochondrial enzyme activity at 37°C (Table 2). Percent growth inhibition was calculated according to the formula: $\triangle$% inhibition = (OD or ct/min of control − OD or ct/min of VH4-34 MoAb/OD or ct/min of control) × 100. In six experiments MoAb 216 inhibited thymidine uptake by a mean of 55·5 ± 6% and MTT metabolism by 47 ± 5·4%. MoAb A6(H4C5) inhibited thymidine uptake by 37·5 ± 5·5% and MTT metabolism by 24 ± 3·6%.

To study the effect of temperature on the cytotoxicity of the two MoAbs, human B cell lines and splenic mononuclear cells were incubated with MoAb 216 at 4°C or 37°C, and analysed on FACS for PI uptake. For every cell type, killing was greater at 4°C than at 37°C (Fig. 3a).

Cytotoxicity of 216 and A6(H4C5) does not appear to require metabolic activity

Greater cytotoxicity at 4°C than at 37°C and the rapidity of MoAb action suggest that active metabolic processes were not required for cell killing. We thus tested cytotoxicity of the two MoAbs in the presence of metabolic inhibitor NaAz. Presence of NaAz did not

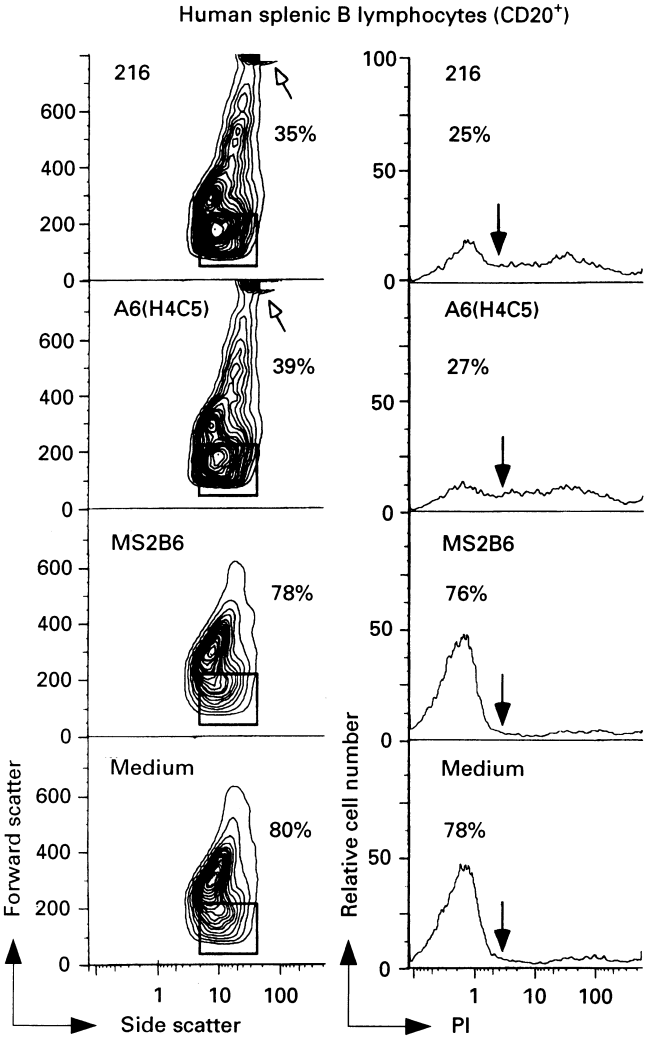

Fig. 1. Cytotoxicity of splenic B cells by VH4-34 MoAbs 216 and A6(H4C5). Splenic mononuclear cells suspended in PBS with 3% fetal calf serum (FCS) at 1×10^6/ml were incubated with human VH4-34 or isotype-matched control MoAbs (10 μg/ml) at 4°C for 1 h, stained with FITC-conjugated anti-CD20 (a pan human B cell marker) and propidium iodide (PI), and then analysed by FACS. The left panel shows scatter contour plots after gating for B cells by anti-CD20. The box shows the scatter signals of dead cells compared with the scatter of viable cells (above the box). The percentage of viable cells in each sample is shown. Human splenic mononuclear cells always have a certain percentage of dead cells after liquid N_2 storage. The open arrow demonstrates the scatter of aggregated cells due to MoAb cross-linking. The right panel shows the PI fluorescence of $CD20^+$ B cells. The percentage of viable cells (PI^-) is shown with each histogram. The arrow in the histograms defines the cut-off gate from channel zero fluorescence for measuring PI^- viable cells. FACS analysis was performed on a dual laser FACS II. Discrimination of live *versus* dead cells by scatter changes has been described in detail and is widely used to gate out dead cells in routine FACS analysis [42]. Dead cells give lower signals in forward light scatter and larger signals in side scatter measurements than the corresponding live cells. The differences in scatter signals from dead and live cells makes it possible to define a region in the forward *versus* side display that includes the live cells and excludes most dead cells and debris. Dead cell identification can be even stronger using PI, that binds to nucleic acids and is effectively excluded from mammalian cells with intact membranes. Since gating samples is easier with PI, and since PI is a better indicator of cell death than scatter signals [42], PI was used for measuring dead cells in all experiments described below.

Table 1. VH4-34-encoded MoAbs 216 and A6(H4C5) bind and kill human B cell lines

	Mean fluorescence*				Percent viable cells $(PI^-)^\dagger$			
	216	A6(H4C5)	MS2B6	Medium	216	A6(H4C5)	MS2B6	Medium
B cell lines								
Nalm-6	525	265	6	5	12	32	96	97
OCI-LY8	495	155	5	4·5	16	52	95	96
Arent	425	101	6	6·5	20	63	99	97
SUP-B8	368	32	3	3·5	29	71	98	99
Enzyme-treated Nalm-6‡	250	NT	5	5	62	NT	97	98
T cell lines								
PEER	1·5	1·7	1·8	1·2	99	98	99	99
HuT 78	5·8	5·2	4·5	4·6	97	96	99	98
Splenic B cells§	450	388	6	7	10	38	89	90

*Presented as the logarithmic scale value of the mean channel number. Fluorescence intensity of propidium iodide $(PI)^-$ cells detected using biotinylated MoAbs (1 μg/ml) with Texas-red strepavidin.

†Cytotoxicity was measured on FACS by PI uptake. Cells were incubated at 4°C for 1 h with MoAb concentrations of 10 μg/ml.

‡Nalm-6 cells were incubated overnight with enzyme endo-β-galactosidase (50 mU) and processed for staining as described. Enzyme treatment did not change the fluorescent intensity of surface antigens CD19 and CD71.

§Data on the binding are presented on B lymphocytes gated with FITC-conjugated anti-CD20. No cell death was seen in the $CD20^-$ population (T cells, monocytes and natural killer (NK) cells) of human splenic MNC. Data are presented from one spleen, five other spleen specimens gave comparable results.

alter the toxicity at either temperature, confirming that killing by the two MoAbs appears to be mediated by an energy-independent mechanism (Fig. 3b). Furthermore, EDTA did not interfere with cytotoxicity at 37°C, suggesting that an initial Ca^{2+} influx did not play a role in the killing process. Cytotoxicity was not due to residual complement activity in the heat-inactivated FCS, as killing took place in Iscove's media without FCS and in PBS with 2% bovine serum albumin (BSA) (Fig. 3b). 216, A6(H4C5) and other isotype-matched human MoAbs used as controls were all prepared under similar laboratory conditions and were extensively dialysed to ensure the absence of unidentified toxic media components. The DNA extracted from B cell lines incubated with MoAb 216 and A6(H4C5) did not show the typical fragmentation associated with apoptosis (data not shown).

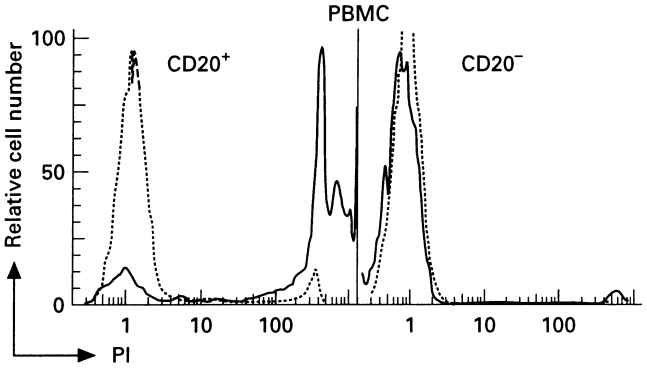

Fig. 2. Cytotoxicity of MoAb 216 is specific for B lymphocytes. Human peripheral blood mononuclear cells (PBMC) at 1×10^6/ml in PBS with 3% fetal calf serum (FCS) were incubated with 216 or isotype-matched control MoAb, MS2B6 (10 μg/ml) at 4°C for 1 h, stained with FITC-conjugated anti-CD20 and propidium iodide (PI), and analysed by FACS. PI profiles of $CD20^+$ and $CD20^-$ cells are shown. The non-dotted histograms are of cells treated with MoAb 216 and the dotted histograms are of cells treated with MoAb MS2B6. FACS analysis was performed on a FACStar^{PLUS}.

Electron microscopy

Scanning electron microscopy (SEM) demonstrated large holes or pores on the surface of cells incubated with the two VH4-34 MoAbs (Fig. 4A,B,E). Cells incubated with control MoAb MS2B6 or untreated cells did not exhibit this morphology (Fig. 4C,D,F). These cells have membrane contours and microvilli typical of lymphoid cells, whereas cells exposed to VH4-34 MoAbs have a more convoluted surface and appear to have a film of debris surrounding the membrane, probably representing cytoplasmic leakage from the pores. In fact, some cells in the samples treated with MoAb 216 appear to be in the process of lysis or disintegration. Generally, a single pore was seen on individual cells. The plasma membrane defect generated is much larger than

Table 2. MoAbs 216 and A6(H4C5) inhibit DNA replication and mitochondrial metabolism in Nalm-6 cells

	^{3}H-thymidine uptake* (ct/min)	MTT (OD at 540 nm)
216	11 740 ± 1120	0·460 ± 0·004
A6(H4C5)	17 203 ± 1880	0·596 ± 0·01
MS2B6	26 014 ± 3710	0·730 ± 0·011
Medium	27 168 ± 2700	0·755 ± 0·006
NaAz (0·05%)	960 ± 220	0·289 ± 0·009

*Pre-B cell line, Nalm-6 in Iscove's with 10% fetal calf serum (FCS) was incubated with 10 μg/ml MoAbs (216, A6(H4C5) and MS2B6) for 18 h at 37°C as described. Experiment shown is a representative of six assays. NaAz, which inhibits metabolic activity, was used as a positive control in both assays.

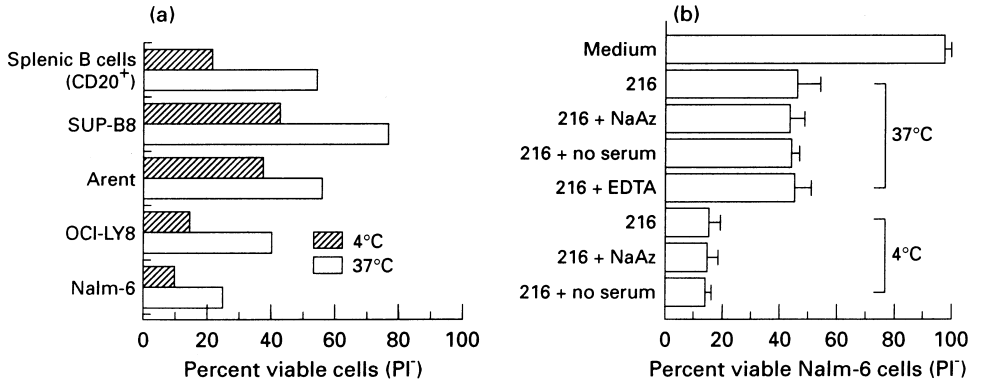

Fig. 3. (a) Cytotoxicity of MoAb 216 is greater at 4°C than at 37°C. Splenic mononuclear cells and B cell lines in PBS with 3% fetal calf serum (FCS) were incubated with 216 (10 μg/ml) at either 4°C or 37°C for 1 h. The cells were then stained with propidium iodide (PI) and analysed on FACS. Percentage of viable PI^- cells for each cell type at the two temperatures is shown. ▨, Cells incubated at 4°C; □, cells incubated at 37°C. All cells incubated with isotype-matched control MoAb or maintained in medium alone for 1 h at either temperature were > 98% viable (data not shown). (b) NaAz (0·05%) and EDTA (30 mM) do not block the cytotoxic effect of MoAb 216. Nalm-6 cells in Iscove's with or without 3% FCS were incubated with 216 (10 μg/ml) at either 4°C or 37°C in the presence of NaAz or EDTA for 2 h. The cells were then stained with PI and analysed on FACS.

the usual ion transport channel or pores formed by staphylococcal α-toxin, *Escherichia coli* haemolysin, the C9 complement component or perforin [26].

DISCUSSION

We describe here a novel mechanism of cell death caused by human MoAbs that is different from both complement-mediated necrosis and energy-dependent apoptosis. Cytotoxicity and binding by MoAbs 216 and A6(H4C5) were B cell-specific. The two MoAbs did not bind or kill T cells in human peripheral blood and spleen or T cell lines HuT 78 and PEER. Cytotoxic effect of the two MoAbs occurred rapidly in the presence of metabolic inhibitor NaAz, suggesting *de novo* protein synthesis was not required for the killing action. Cell death was reflected as a decrease in dividing and metabolizing cells in the thymidine uptake and MTT assay, respectively. Large membrane pore-like structures, that probably lead to cellular leakage and death, were observed within 15 min of MoAb exposure under SEM. PI^+ cells were observed within 5 min of MoAb treatment.

Recently two other studies reported MoAbs that appear to mediate rapid cytotoxicity similar to MoAbs 216 and A6(H4C5). Matsuoka *et al.* describe a rat MoAb RE2 that kills T cell clones in a complement-independent manner [10]. MoAb RE2 is cytotoxic within minutes of exposure, and similar to our MoAbs its activity is not inhibited by EDTA and azide. Moreover, RE2 induces formation of giant membrane holes that appear very similar to the pores formed by MoAbs 216 and A6(H4C5). Unlike our two human MoAbs, however, cytotoxicity of MoAb RE2 is greater at 37°C than at 4°C [10]. The second study describes a mouse MoAb BR96 that mediates rapid PI uptake at 4°C in the presence of azide [27,28].

MoAbs BR96 and RE2 are IgG in isotype, whereas 216 and A6(H4C5) are human IgMs. The epitopes recognized by these MoAbs are distinct. Rat MoAb RE2 is believed to react with an unique epitope associated with mouse class I antigen [10]. Murine MoAb BR96 binds to the Le^y epitope which is increased on lysosomal membrane proteins in some carcinomas [29]. 216 and A6(H4C5) are human MoAbs that bind to a carbohydrate epitope on B lymphocytes similar in structure to the i antigen present on cord erythrocytes [6,7,9]. Further identification and/or immunoprecipitation of the human B cell antigen after membrane detergent extraction of B cell lines has thus far been unsuccessful. As these different MoAbs (rat, mouse and human) recognize different epitopes, probably on different cell structures, the possibility exists that this novel form of cell death is caused by the structure of the MoAb. Studies with MoAb RE2 suggest involvement of cytoskeletal structures for cytolysis [10]. We are at present investigating the role of cell cycle, extracellular matrix and cytoskeletal structure for 216 and A6(H4C5) action.

This is the first study describing complement-independent cytotoxicity of B lymphocytes by CAs. Previous studies have described complement-dependent lymphocytotoxic CAs that kill both T and B lymphocytes [30–33]. Our preliminary analysis shows that complement-independent cytotoxicity is not restricted to MoAbs 216 and A6(H4C5), as seven other independently derived VH4-34 MoAbs also cause PI uptake in human B lymphocytes within 5–10 min of exposure (manuscript in preparation).

In normal adults, the level of VH4-34-encoded antibodies is low [34]. However, VH4-34 antibody levels are routinely found to be elevated under certain pathological/infectious conditions [1,2,35–37]. Furthermore, MoAbs encoded by the VH4-34 gene have been shown to be over-represented within the autoimmune repertoire [38] and there is an increased incidence of VH4-34-expressing malignant B lymphomas [39–41]. This increased expression during disease may be associated with the complement-independent B cell cytotoxicity we report in this study. Further studies investigating the mechanism of pore formation and its relevance to B cell regulation *in vivo* may help explain the role of VH4-34 gene-derived antibodies under immunological stress.

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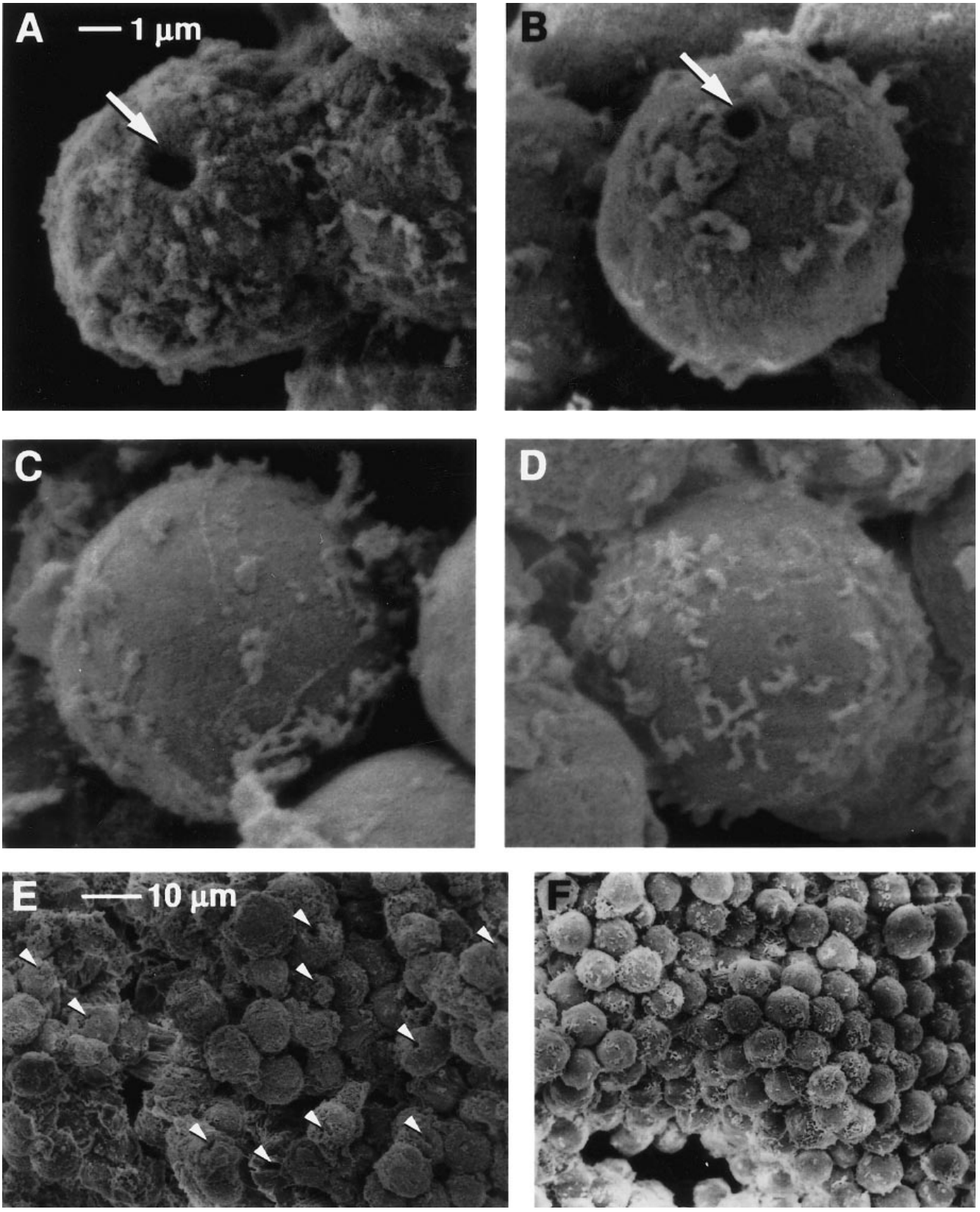

Fig. 4. MoAb 216 and A6(H4C5)-treated Nalm-6 cells develop membrane pores. Cells were incubated with MoAbs 216 (A), A6(H4C5) (B), MS2B6 (C) or kept in medium alone (D) for 15 min at 4°C and then processed as described in Materials and Methods. The arrow demonstrates the position of the membrane pores. The magnification of A–D is the same. Overview of cells treated with MoAbs 216 (E) and MS2B6 (F) shows that multiple cells develop pores when exposed to VH4-34 MoAb 216 (arrowheads) but not with control MoAb. The magnification of pictures E and F is the same. Assuming there is an equal probability of pore formation on the other side of a cell, 50% Nalm-6 cells exhibit pores when treated with MoAb 216 at 5 μg/ml.

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