Clarke, Modet & Cº

COLOMBIA

P-1922

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
DIVISION DE NUEVAS CREACIONES
E. S. D.

Ref.: Expediente No. **06-30455**
Solicitud de Patente a nombre de **Novartis AG**

Yo, DILIA MARIA RODRIGUEZ D'ALEMAN, actuando como apoderada sustituta de acuerdo con la sustitución adjunta, identificada como aparece al pie de mi firma, domiciliada en la Carrera 11 No. 86-53, piso 6, obrando como apoderada de la sociedad **Novartis AG**, domiciliada en Basilea, Suiza, solicitante de la patente de la referencia, en respuesta a su oficio No. 05166 notificado por fijación en lista el día 18 de marzo de 2010, me permito acompañar los siguientes documentos solicitados por ese despacho:

G. AVERSA ET AL.: "A monoclonal antibody (A6) recognizing a unique epitope restricted to CD45RO and -RB isoforms of the leukocyte common antigen family identifies functional T cell subsets." CELLULAR IMMUNOLOGY, vol. 158, no. 2, 15 October 1994 (1994-10-15), pages 314-328, XP002228858 the whole document

G. AVERSA ET AL.: "Use of monoclonal antibodies to study in vivo and in vitro activated lymphocytes." TRANSPLANTATION PROCEEDINGS, vol. 21, no. 1(1), February 1989 (1989-02), pages 349-350, XP008017039 NEW YORK, NY, USA the whole document

---

S. GREGORI ET AL.: "Anti-CD45RO/RB mAb a new tool for tolerance induction."
THE FASEB JOURNAL,
vol. 17, no. 7,
14 April 2003 (2003-04-14), page C63,
XP008046594
BETHESDA, MD, USA
abstract 37.23

D. DUNN-WALTERS ET AL.: "Effect of somatic hypermutation on potential N-glycosylation sites in human immunoglobulin heavy chain variable regions."
MOLECULAR IMMUNOLOGY,
vol. 37, February 2000 (2000-02), pages 107-113, XP002258153
the whole document

Espero con esto haber dado cabal cumplimiento a lo solicitado por ese despacho y de conformidad con lo indicado en el artículo 46 de la Decisión 486 de la Comisión de la Comunidad Andina.

Señor Superintendente,

DILIA MARÍA RODRÍGUEZ D´ALEMAN
C.C. 41.654.882 de Bogotá.
T.P. 20824 CSJ

CELLULAR IMMUNOLOGY 158, 314–328 (1994)

A Monoclonal Antibody (A6) Recognizing a Unique Epitope Restricted to CD45RO and RB Isoforms of the Leukocyte Common Antigen Family Identifies Functional T Cell Subsets[1]

GREGORIO AVERSA,[2] JENNIFER A. WAUGH,[3] AND BRUCE M. HALL[4]

Department of Medicine, University of New South Wales and Liverpool Hospital, New South Wales, Australia

Received October 4, 1993; accepted June 29, 1994

The mAb A6 was produced by immunization of mice with human PHA-stimulated PBMC. Immunoprecipitation studies and staining of cell lines transfected with individual leukocyte common antigen (LCA) isoforms showed that A6 recognizes a unique epitope strongly expressed on the lower MW isoform (p180) of LCA, but also weakly expressed on the p190 isoform coded by exon B and the p205 coded by exons A and B. The epitope recognized by A6 was carbohydrate-dependent in that it was neuraminidase-sensitive, but trypsin-resistant. A6 stained most TCR-$\alpha\beta^+$ cells with differential intensities, subdividing them into a bright and dim population, and strongly stained all TCR-$\gamma\delta^+$ cells. A6 did not stain $CD19^+$ B cells nor $CD56^+$ NK cells. Anti-CD45 mAb such as UCHL1 recognizing CD45RO have been used to define memory T cells. Depletion of PBMC subsets with A6 or UCHL1 mAb dramatically decreased proliferative responses to the recall antigens anti-CD3 mAb and alloantigen and enhanced their responses to PHA. A6, unlike UCHL1, also depleted alloreactive T cells that affect primary and secondary MLC and CML. Thus, A6 was shown to recognize the lower MW isoforms of LCA which are expressed on functional T cell subsets including memory, activated, and alloreactive T cells.

INTRODUCTION

The leukocyte common antigen (LCA)[5] family is composed of a group of high-molecular-weight (180–220 kDa) glycoproteins generated by the alternative splicing of exons 4, 5, and 6 coding for the LCA external domain resulting in the expression of 220-, 205-, 190-, and 180-kDa isoforms (1). Exons 4, 5, and 6 encode determinants

[1] This work was supported by grants from the National Health and Medical Research Council of Australia, the Australian Kidney Foundation, the Lions Club of District 102N2, and NIH Grants AI 26041 and DK 40800. Part of this work was performed in the Division of Nephrology, Stanford University Medical School, Stanford, CA and Department of Nephrology, Royal Prince Alfred Hospital, Camperdown, 2050 NSW Australia. We thank Dr. Michel Streuli for providing the data of the transfectant studies, Dr. P. C. L. Beverley for providing UCHL1, Dr. C. E. Rudd for providing 2H4, and Dr. A. Krensky and Dr. C. Clayberger for the T cell clones.

[2] Current address: DNAX Research Institute, 901 California Ave., Palo Alto, CA 94305.

[3] Current address: Department of Pathology, St. Vincent's Hospital, Fitzroy, Vic. Australia.

[4] To whom correspondence should be addressed at Department of Medicine, P.O. Box 103, Liverpool Hospital, NSW 2170, Australia.

[5] Abbreviations used: LCA, leukocyte common antigen; CML, cell-mediated lympholysis; mAb, monoclonal antibody; PBMC, peripheral blood mononuclear cells.

0008-8749/94 $6.00

termed CD45RA, CD45RB, and CD45RC, respectively, whereas the absence of all three exons results in the generation of the p180 isoform expressing the CD45RO epitope (1). These isoforms are differentially expressed on leukocyte populations (1). Numerous mAb have been developed which recognize common epitopes present on all members of the family (2). Limited numbers of monoclonal antibodies recognizing epitopes restricted to one or more isoforms have been described (3, 4).

The majority of peripheral T cells express either p180 or p220, and a small proportion express both (4, 5). The p190 and p205 isoforms of LCA are also expressed by T cells (1), but their precise identification is limited by the lack of specific mAb. The p220 is expressed by naive T cells, which lose this marker and acquire the p180 following activation and retain it as memory cells (6, 7). The p220 form contains a trypsin-sensitive epitope (CD45RA) that is also present on one of the p205 isoforms coded by exons A and B (5, 8–10). UCHL1, a mAb belonging to the CD45RO cluster (2–4), recognizes a carbohydrate-dependent epitope (10) present exclusively on the 180-kDa form of the LCA molecule that is formed by the directed joining of exons 3 and 7 without the usage of the variable exons A, B, and C (4, 11). Expression of this LCA isoform identified by the mAb UCHL1 is correlated with memory T cell function (3, 7, 12, 13). The $CD45RA^+$ subset defines naive cells that respond to PHA, but not to recall Ag or anti-CD3 mAb (14, 15).

In this manuscript we describe A6,[6] a mAb that recognizes an epitope strongly expressed on the p180 isoform of LCA but also detectable on one of the p190 and p205 isoforms. It is also shown that the expression of the isoforms recognized by the A6 mAb is correlated with T cell responsiveness in MLC, in addition to memory function.

MATERIALS AND METHODS

Source of Cells and Tissues

PBMC were obtained from normal donors. Normal spleen, kidney, thymus, and liver were obtained from cadaver transplant donors and rejecting renal and liver biopsies were obtained for clinical diagnosis. The sources of cells used were baboons (*Papio hamadrya*) and macaque (*Macaca fascicularis*) lymphocytes (Animal House R.P.A. Hospital, NSW Australia); mouse cell lines NS-1 and L929 (Dr. R. Raison, CIRCUS University of Sydney); Molt-4, Namalwa, JP, (Ludwig Institute, University of Sydney, Australia); RC2a, U-937, HL-60 (Dr. C. Geczy, Kolling Institute, R.N.S. Hospital, St. Leonards, NSW Australia); and CCRF-CEM, HPB-ALL, HSB, HUT-78, PEER, Jurkat, the alloreactive T cell clones APL 2.1, AMS B.2 and AMS H.10, and the cell line AJY (Drs. C. Clayberger and A. Krensky, Dept. of Pediatrics, Stanford) (16).

Production of the Hybridoma

The hybridoma cell line secreting A6 was generated by the standard hybridoma technique (17) with modifications as described (18), fusing NS-1 cells with splenocytes from a BALB/c mouse (Blackburn Animal House, University of Sydney) immunized with PHA-stimulated human pooled PBMC. A6 was found to be IgGlk by Misotest enzyme immunoassay (CSL, Parkville, Vic. Australia).

[6] At the recent Fifth International Workshop on Leukocyte Differentiation Antigens, A6 was designated a unique "CD45RO-like" cluster.

4

Indirect Immunofluorescence Staining of Cells

Standard techniques were used for one- and two-color staining using fluorochrome-labeled mAbs or unlabeled mAbs followed by sheep anti-mouse Ig-FITC (Silenus, Hawthorne, Vic. Australia) or biotin-conjugated mAbs followed by streptavidin-PE (Becton Dickinson, San Jose, CA). The cells were analyzed with a FACS IV or a FACScan (Becton Dickinson). Anti-CD45 mAb used included UCHL1 (CD45RO, 180-kDa isoform) (2, 3), a gift of Dr. P. C. L. Beverley (ICRF Tumour Immunology, London, UK); UCHL1-FITC (Pharmingen, San Diego, CA); 2H4 (CD45RA, 220- to 205-kDa isoforms) (2), a gift of Dr. C. E. Rudd (Dana Farber Cancer Institute, Boston MA); DAKO LCA (2B11, 220-, 190-, and 180-kDa isoforms) (2); PD 7/26 CD45RB (220-, 205-, and 190-kDa isoforms; purchased from DAKOPATTS Glostrup, Denmark) (2); and G22.4 and C76.5 (CD45 mAbs were a gift of Dr. C. Clayberger, Dept. of Cardiac Surgery, Stanford). CD45 mAb 4.14, reactive with a carbohydrate-dependent epitope present on some members of LCA, was produced in our laboratory (unpublished data). FITC- or phycoerythrin (PE)-conjugated anti-Leu-18 (CD45RA), anti-HLe-1 (CD45), anti-Leu-12, anti-Leu-19, anti-TCR-1, anti-TCR-$\gamma\delta$1, anti-Leu-3a (CD4), anti-Leu-2-PE (CD8), anti-Leu-4-PE (CD3), control mouse Ig-PE, and control mouse Ig-biotin were all purchased from Becton Dickinson. RPA-T4 (CD4) and RPA-T8 (CD8) were produced in our laboratory (19, 20). For double immunofluorescence and blocking studies A6 was conjugated with FITC or biotinylated as described (21). Transfected murine cell lines were stained as described (4).

Isolation of Cell Populations

PBMC were isolated by centrifugation over Ficoll (Pharmacia, Uppsala, Sweden). Depletion of T cell subsets was performed using a previously described panning technique (22), using A6, RPA-T4 and RPA-T8, and MRC-OX8 (specific for rat CD8; a gift of Dr. A. Williams, MRC Cellular Immunology Unit, Oxford, UK) as a control. The panned cells were analyzed by flow cytometry.

Immunoperoxidase Staining of Tissue Sections

A previously described four-step indirect immunoperoxidase technique was used (23).

Radioiodination and SDS–PAGE

The HLA-A2-specific T cell clone AMS H10 and PBMC were radiolabeled with ^{125}I using the lactoperoxidase-catalyzed reaction as described (24). SDS–polyacrylamide gel electrophoresis was performed using a modification of the Laemmli procedure (25), using full-size custom-made gels (7% acrylamide) or minigels (10% acrylamide; Integrated Separation Systems, Netick, MA). Autoradiography was performed using Kodak X-AR-5 film and a Kodak X-Omatic C2 cassette with Lanex intensifying screens (Kodak, Sydney, Australia). A PhosphorImager (Molecular Dynamics, Menlo Park, CA) was used to analyze the minigels after exposure of these to the screen of a PhosphorImager cassette.

Protease and Glycosidase Treatments

Freshly isolated (Ficoll) PBMC were washed four times and resuspended in PBS at 5×10^6/ml, treated for 1 hr at 37°C with neuraminidase (Boehringer–Manheim,

Indianapolis, IN), trypsin (Sigma Chemical Co., St. Louis, MO), protease (Sigma), or left untreated. One milliliter of FCS was added at the end of the incubation, and the cells were washed three times in PBS and stained by indirect immunofluorescence with various mAb. The cells were analyzed with a FACScan (Becton Dickinson, San Jose, CA).

Proliferation and CML Assays

PBMC depleted of cell subsets by panning were resuspended at 1×10^6/ml in RPMI 1640 (Irvine Scientific, Irvine, CA), supplemented with 10% human AB serum (Irvine), penicillin, and streptomycin, and dispensed in U-bottom 96-well trays containing one of the following: tetanus toxoid at 1:4000 (Department of Health Massachusetts, Boston, MA.), 50 ng/ml recombinant hepatitis B antigen (Merck, Sharp & Dohme, West Point, PA), 5 μg/ml PHA (Sigma), or 10 ng/ml purified OKT3 produced from ATCC cell line. Proliferation was assessed 72 hr later by thymidine incorporation (26). MLC-CML was assayed using the standard method (26). Allogenic stimulators were irradiated with 3000 rads from an X-ray source (Philips) and target cells for CML were Con A stimulated PBMC blasts. CTL activities of whole and the subset-depleted MLC populations were assessed by ^{51}Cr release of lysed specific allogeneic target cells. ^{51}Cr release was measured with the use of a gamma counter and the percentage target cell lysis (Y axis) was calculated as

$$\% \text{ Lysis} = \frac{\text{ER} - \text{SR}}{\text{TR} - \text{SR}} \times 100,$$

where ER is the experimental release, SR is the spontaneous release, and TR is the total release.

RESULTS

Biochemical Analysis of the Antigen Precipitated by A6

Figure 1A compares the molecules immunoprecipitated by A6 (lane 4) with those precipitated by DAKO LCA mAbs PD7 and 2B11 (lane 2), UCHL1 (lane 3), 2H4 (lane 5), and other CD45 mAb (lanes 6, 7, 8) from radiolabeled cell lysates of a cytotoxic $CD8^+$ T cell clone (AMS H10). A6 precipitated a molecule forming a dense band around 160–170 kDa and extending into the 180-kDa region (lane 4). The former band was similar to that precipitated by UCHL1. The 4.14 (lane 6) and C76.5 (lane 8) precipitates formed two major bands at 190 and 180-kDa. DAKO LC (lane 2) precipitated a 180-kDa molecule. No major bands were detected by immunoprecipitation with 2H4 (lane 5) or the control mAb (lane 9). These results are consistent with the mAb reactivity with AMS H10, an alloreactive T cell clone, that stained with the mAb tested except 2H4, showing it only expresses the lower MW isoforms of LCA consistent with its activation state.

Following T cell activation, other proteins associate with CD45 and are precipitated, as described (27–30). Two fainter bands were present in the 130- and 120-kDa areas with A6, G22.4, and 4.14 and only at 130 kDa with DAKO LC and at 120 kDa with UCHL1. No bands were observed in these regions in the 2H4 or control mAb lanes. Additional bands at 80 and 65 kDa were precipitated by A6, G22.4, and C76.5. Bands were also present at ~46 kDa, but most of them were nonspecific as they were present in all lanes, including the controls, although one could be CD2 known to associate

6

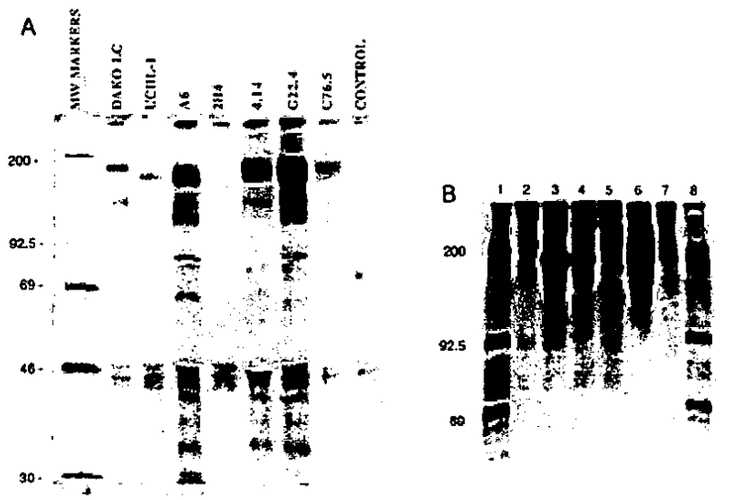


FIG. 1. (A) SDS–PAGE of molecules precipitated from ^{125}I-labeled AMS H10 T cell line by various mAb. Immunoprecipitation was performed with SaC cells coated with mAbs as indicated. Autoradiography was performed as indicated under Materials and Methods, by exposing the gel for 24 hr. 1 (B) SDS–PAGE of molecules precipitated from ^{125}I-labeled PBMC, under reducing conditions. Lanes 1 and 8, MW markers; lane 2, control rabbit anti-mouse Ig-coated SaC; lane 3, A6; lane 4, UCHL1; lane 5, 2H4; lane 6, anti-HLe-1; lane 7, 4.14 mAb. All lanes represent overnight exposure to a screen of a PhosphorImager cassette, except for lane 6 which represents a 3-hr exposure.

with CD45 (27). A prominent 33-kDa band was identified and this is consistent with CD8 (28) and another band at ~30 kDa. These bands were seen with all anti-LCA mAb but were most prominent with A6, G22.4, and 4.14.

Preclearing of AMS H10 cell lysates with G22.4, an unrestricted CD45 mAb, abolished the subsequent precipitation of proteins with either A6 or UCHL1 (data not shown). Preclearing with A6 or UCHL1 greatly reduced protein precipitation with A6 and UCHL1 (data not shown). These findings indicated that the Ags recognized by A6 are also members of the LCA family and include the p180 isoform also recognized by UCHL1.

Both A6 and UCHL1 precipitated a single molecule of about 180 kDa from radiolabeled cell lysates of PBMC (Fig. 1B), consistent with the lower MW isoform of LCA. As expected, the CD45RA mAb 2H4 precipitated a molecule of about 220 kDa, whereas the CD45 mAb anti-HLe-1 precipitated several molecules spanning the whole 180- to 220-kDa range, consistent with all isoforms of LCA (Fig. 1B). The mAb 4.14 precipitated molecules in the region of 180 and 200–210 kDa, consistent with the lower and intermediate isoforms of LCA.

A6 Staining of Mouse Cells Transfected with Individual Human LCA Constructs

A6 was tested on a panel of mouse pre-B 300-19 cells transfected with individual human LCA constructs (4, 9). Staining of these cells with A6 was compared to that of UCHL1 and GAP 8.3, a CD45 mAb that reacts with all LCA isoforms (Fig. 2). Both A6 and UCHL1 strongly stained LCA.1, the cell line expressing the p180 (CD45RO) product of the construct which does not use any of the variable exons. A6

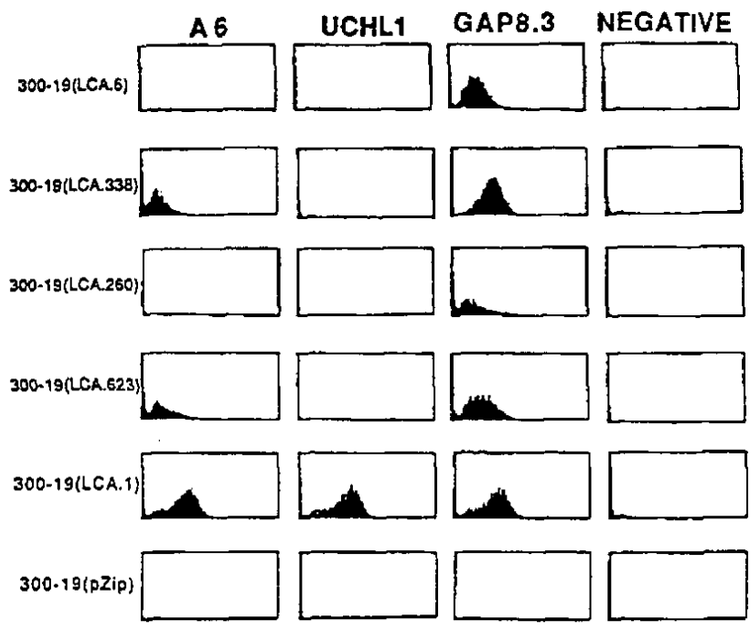


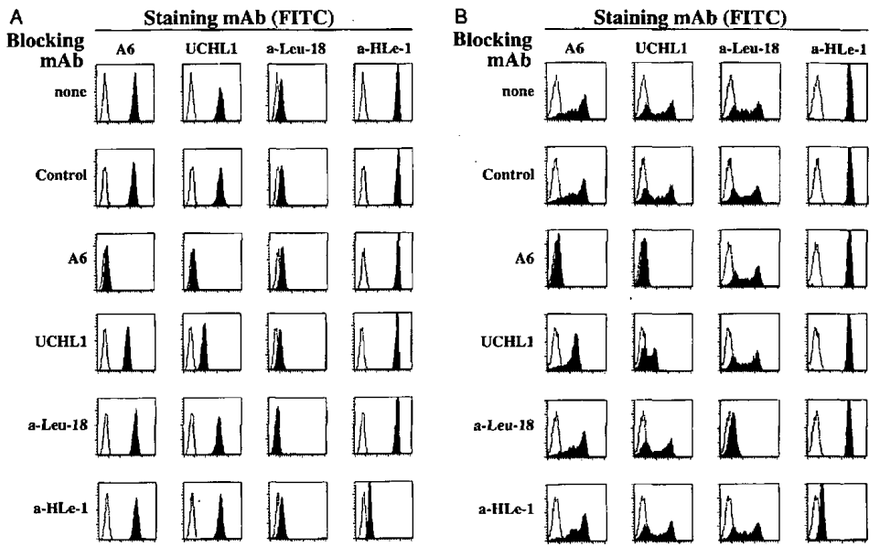
FIG. 2. Cytofluorographic analysis of murine pre-B cell line expressing individual human leukocyte common antigen isoforms as previously described (4, 34). All mAb were in ascites form except for the negative control, which was purified mouse Ig (5 μg/stain). The ascite dilutions were as follows: GAP 8.3 = 1:400; UCHL1 = 1:300; A6 = 1:800. The cells were stained by indirect immunofluorescene and analyzed with an Epics V flow cytometer (Coulter Electronics, Hialeah, FL). The *X* axis shows the fluorescence intensity (Log scale), the *Y* axis shows the cell number (Log scale; those data were generously provided by Dr. M. Streuli).

also weakly stained LCA.623, expressing a p190 isoform coded by exon B, and LCA.338, a p205 construct containing exons A and B. This latter weak staining was not found with UCHL1.

A6 and UCHL1 did not stain the p220-expressing cell line LCA.6 nor LCA.260, the cell line expressing the construct which uses exons B and C. These studies indicated that A6 bound an epitope that was not identical to that bound by UCHL1.

A6 Blocks the Binding of UCHL1 (CD45RO), but Not CD45RA or CD45 mAb

Preincubation of a T cell clone (Fig. 3A) or PBL (Fig. 3B) with A6 totally blocked the binding of FITC-conjugated UCHL1 or A6. Preincubation with UCHL1 partially blocked A6-FITC binding (but also did not completely block the binding of UCHL1-FITC), indicating that the two mAb recognize the same or close epitopes but that the binding affinity of A6 was much greater. Binding of the CD45 mAb anti-HLe-1 and the CD45RA mAb anti-Leu-18 was not affected by pretreatment of the cells with A6 or UCHL1, whereas their binding was almost completely blocked by unlabeled anti-HLe-1 and anti-Leu-18, respectively (Figs. 3A and 3B). These findings indicate that the epitopes recognized by A6 and UCHL1 are very closely located.


A
Staining mAb (FITC)
Blocking mAb
A6
UCHL1
a-Leu-18
a-HLe-1
none
Control
A6
UCHL1
a-Leu-18
a-HLe-1
B
Staining mAb (FITC)
Blocking mAb
A6
UCHL1
a-Leu-18
a-HLe-1
none
Control
A6
UCHL1
a-Leu-18
a-HLe-1

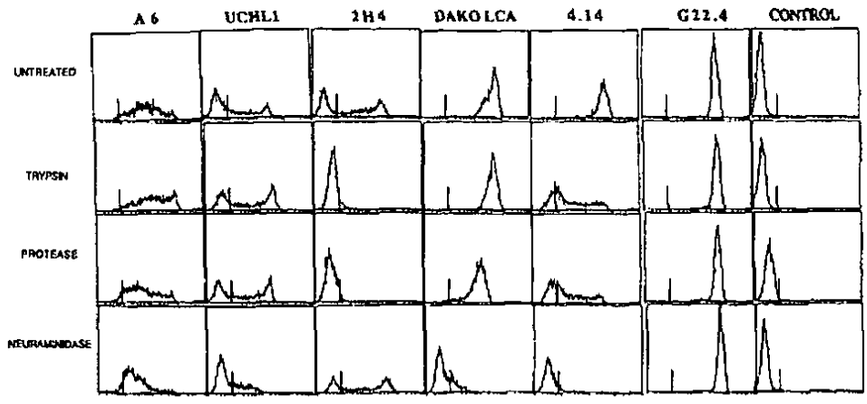


FIG. 4. Enzyme sensitivity of the epitope recognized by A6 and other LCA mAb. Normal PBMC were treated for 1 hr at 37°C with trypsin, protease, or neuraminidase or left untreated and then stained by indirect immunofluorescence with various mAb as described under Materials and Methods. The cells were analyzed with a FACScan.

Effect of Enzyme Treatment on A6 Bindings

Treatment of normal PBL with neuraminidase decreased the intensity of A6 and UCHL1 stains and virtually abolished DAKO LC staining, but had no effect on 2H4 staining (Fig. 4, fourth row). Trypsin treatment removed 2H4 reactivity, but increased the staining with A6 or UCHL1 (Fig. 4, second row). Protease treatment moderately decreased A6, DAKO LC, and to a lesser extent UCHL1 reactivities while it abolished 2H4 staining (Fig. 4, third row). These data suggested that A6 and UCHL1 epitopes are at least in part carbohydrate dependent.

Staining of Cell Populations with A6

Staining of normal PBL with A6 by indirect immunofluorescence and FACS analysis showed three populations with most donors: a negative, a dim, and a bright population. A6 and 2H4 stained mostly reciprocal populations, although double-positive cells were seen (Fig. 5), a pattern also reported for UCHL1 and 2H4 (5). The proportion of cells stained by A6 also varied between normal individuals. All UCHL1$^+$ cells stained with A6 (25–40%) and the majority had high UCHL1 and A6 expression. There was a subpopulation that had intermediate to low staining with A6 (40–55%) that was UCHL1$^-$ but some of which (approximately 15%) was 2H4$^+$. 2H4$^+$, A6$^-$ cells constituted 15–25% of cells. A6 also bound to most CD3$^+$ and TCR-$\alpha\beta^+$ cells, while it strongly stained all the TCR-$\gamma\delta^+$ cells (Fig. 5). A6 did not stain CD19$^+$ (Leu-12) B cells, CD56$^+$ (Leu-19) NK cells (Fig. 5), nor EBV-transformed B cell lines (not shown). A6 stained over 80% of CD4$^+$ cells

FIG. 3. Blocking of fluorescinated mAbs binding to a T cell clone or PBL by unlabeled mAb. Cells from B21 CD4$^+$ T cell clone (A) or PBL (B) were incubated with oversaturating concentrations of mAbs for 1 hr at 4°C and then stained with FITC-conjugated mAb as indicated and analyzed by a FACScan. The solid histograms represent the staining with the test mAb, and the dotted histograms represent staining with a control mAb.

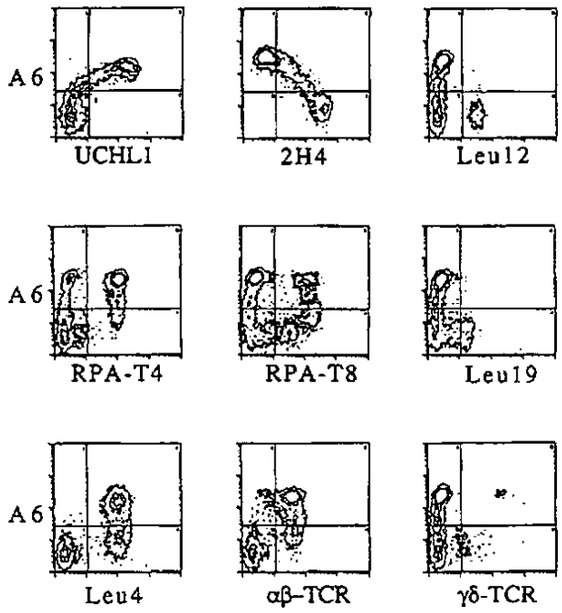


FIG. 5. Two-color analyses of PBL stained with A6 and other mAb. 10^6 PBMC were stained by indirect immunofluorescence with the various mAb and goat anti-mouse Ig-FITC. Biotinylated A6 was added followed by avidin-PE as described under Materials and Methods and then analyzed with a FACScan; the ordinate shows the A6 staining (red fluorescence).

and 60–70% of $CD8^{high}$ cells (Fig. 5). A6 also stained monocytes (90–96%) and to a lesser extent granulocytes (55–99%). When PBL were activated with PHA or in MLC, nearly all cells (80–97%) from all donors stained brightly with A6, showing a similar profile to the one observed with UCHL1 (not shown). A6 also stained 20% of macaque PBL and monocytes, but it stained neither resting nor PHA-stimulated baboon PBMC.

A6 stained four of six human tumor T lymphoblastoid lines, including CEM, HSB, Jurkat, and MOLT 4 but not HPB-ALL, HUT-78, or the TCR-$\gamma\delta^+$ line PEER (Table 1). One difference between A6 and UCHL1 was that UCHL1, as previously described (3), did not stain the Jurkat cell line, but A6 did. In other experiments Jurkat lines from different laboratories had low UCHL1 expression never as high as A6. This suggested that this line may express high levels of p190 but low levels of p180. A6 also stained a promyelocytic line (HL-60) and one of two monocytic lines (RC2a not U937). All three long-term alloreactive T cell clones (APL 2.1, AMSB2, and AMSH10) and the alloreactive T cell line AJY stained with A6.

In normal thymus A6 stained nearly all cortical thymocytes and about half of the medullary thymocytes, a similar pattern to that we observed with UCHL1. In lymph nodes, A6 stained the majority of cells in paracortical areas and occasional cells in follicles. In spleen, A6 stained the majority of cells in the periarteriolar lymphoid sheath. In normal kidney A6 stained few dendritic cells in intertubular areas, and in liver it stained Kupffer cells and some tissue macrophages (not shown). In rejecting renal and liver (not shown) allografts nearly all infiltrating

TABLE 1

Reactivity of A6 with Human Cell Lines and Clones

Cell line	Type	% Cells staining with A6
Tumor cell lines		
CCRF-CEM	T	100
HPB-ALL	T	0
HSB	T	92
HUT-78	T	1
Jurkat	T	99
MOLT-4	T	92
PEER	T (TCR-$\gamma\delta^+$)	100
NAMALWA	B	3
JP	B	60
RC2a	Monocyte	93
U937	Monocyte	2
HL-60	Promyelocyte	90
Alloreactive T cell line and clones		
AJY	T cell line ($CD8^+$)	98
APL 2.1	T cell clone ($CD4^+$)	97
AMS B2	T cell clone ($CD8^+$)	93
AMS H10	T cell clone ($CD8^+$)	99

Note. The cells were labeled with mAb followed by sheep anti-mouse Ig-FITC and analyzed by flow cytometry. The values are the corrected (minus control) percentages of cells stained with A6. Control staining was in the range of 0.5–2.5%.

mononuclear cells stained with A6. A6 also stained T cells in paraffin-fixed tissues as described (31).

Proliferative Responses of $A6^{high}$ and $A6^{low}$ Cells to Recall Ag, Mitogenic CD3, and PHA

Freshly isolated PBMC from donors sensitized to tetanus toxoid and hepatitis B were depleted of cell subsets using A6, UCHL1, or 2H4 mAb in a panning technique. The populations of cells remaining after depletion with A6 and UCHL1 were analyzed by FACS. A6-depleted cells (herein referred to as $A6^{high}$-depleted cells) were 50–60% $CD3^+$, ~30% $CD4^+$, ~65% $CD45RA^+$, and <5% $CD45RO^+$ (UCHL1) and contained ~20% $A6^{low}$ cells but <5% $A6^{high}$ cells. UCHL1-depleted cells were 70–80% $CD3^+$, ~50% $CD4^+$, ~55% $CD45RA^+$, and <5% $UCHL1^+$ and contained ~70% $A6^{low}$ cells but <5% $A6^{high}$ cells.

These subset-depleted cells were cultured for 4 days with tetanus toxoid, recombinant hepatitis B antigen, and mitogenic concentrations of OKT3 or PHA. Figure 6a shows a representative experiment, in which depletion of PBMC $A6^{high}$ or $UCHL1^+$ populations markedly reduced their proliferative responses to both recall antigens. Depletion of the complementary $2H4^+$ subset increased cell proliferation, indicating that the $A6^{high}$ and the $UCHL1^+$ subsets contain the memory cells. Depletion of $A6^{high}$ or $UCHL1^+$ cells markedly reduced OKT3-induced proliferation, and depletion of $2H4^+$ cells enhanced proliferation probably by enriching the memory cell concentration (Fig. 6b). Control panning with irrelevant MRC-OX8 also moderately increased proliferation compared to untreated controls, showing that the panning procedure did

12

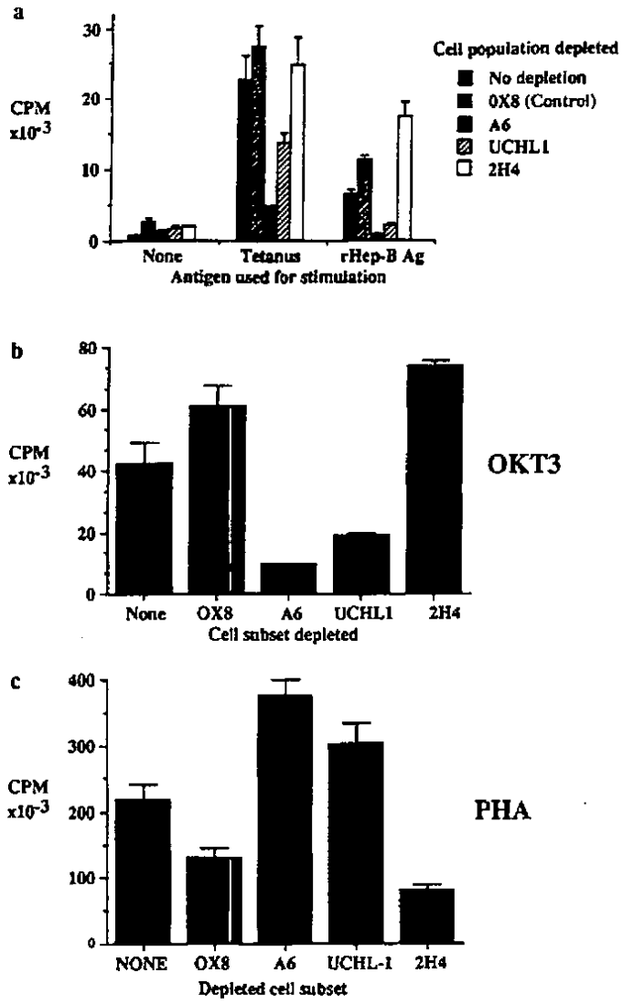


FIG. 6. (a) Proliferation to recall antigens of PBMC after depletion with specified mAb. (b and c) Proliferation in response to polyclonal mitogens, OKT3, or PHA. The *Y* axis shows the beta counts per minute (cpm). The bars represent the mean cpm of triplicate wells; the error bars are the standard deviations.

not account for the reduced proliferation to OKT3 of populations depleted of $A6^{high}$ cells (Fig. 6). PHA-induced proliferation showed converse results, with $2H4^+$-depleted populations having diminished responses and $A6^{high}$- and $UCHL1^+$-depleted populations having increased responses (Fig. 6c). Thus, A6 was shown to recognize a functional subset which includes memory T cells.

Alloreactive T Cells are Contained in the $A6^{high}$ Population

Depletion of $A6^{high}$ cells markedly reduced primary MLC responses, whereas, in agreement with previous reports (12), depletion of $UCHL1^+$ cells had no effect on

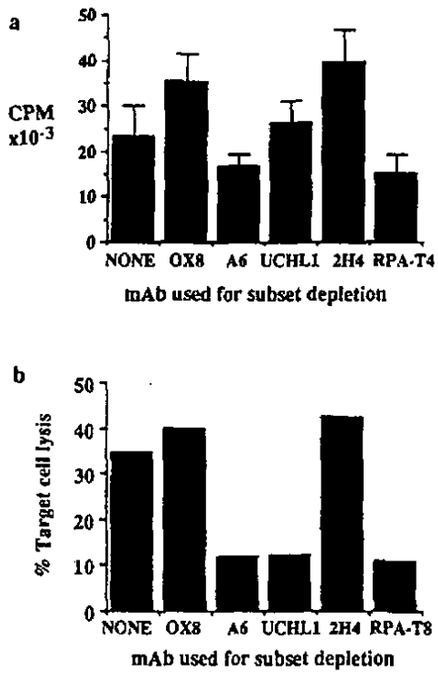


FIG. 7. (a) Primary MLC proliferative responses of PBMC depleted of various subsets. PBMC were depleted by panning with the indicated mAb. Counts represent means and standard deviations of triplicate cultures. (b) Specific CTL activity of alloreactive T cells generated in MLC and depleted of cell subsets. Various subsets (*X* axis) were depleted from a 7-day MLC by panning with mAb, and the remaining cells were used as effector cells in CML against specific PHA-stimulated allogeneic target cells in a standard ^{51}Cr release assay.

alloreactivity nor did depletion of $2H4^+$ cells (Fig. 7a). Addition of A6 to MLC did not alter the proliferative responses (data not shown). In secondary MLC, response rechallenge with specific donor antigen was greater than with third party. Depletion of $A6^{high}$ or $UCHL1^+$ cells but not the $2H4^+$ subset markedly reduced responsiveness to both specific and third-party stimulators.

Removal of $A6^{high}$ or $UCHL1^+$ cells from a 7-day MLC dramatically reduced CTL activity against ^{51}Cr-labeled specific target cells to a degree comparable to that of $CD8^+$ cell depletion (Fig. 7b). Enrichment of the $A6^{high}$ population by removal of the complementary $2H4^+$ subset enhanced CTL activity, which showed that most if not all CTL generated are $A6^{high}$. However, addition of A6 mAb to cultures prior to CML assays did not alter specific or third-party target cell lysis (not shown), indicating that the epitope recognized by A6 is not directly involved in the CTL effector mechanism.

DISCUSSION

Although the molecular mechanisms generating the various LCA isoforms have been elucidated (9, 11, 32), the regulation of expression and functional significance of these glycoproteins is not fully resolved. Although several mAb to CD45RA have been produced (2), so far UCHL1 has been the only mAb identifying an epitope

present exclusively on the p180 isoform of LCA (4), the form expressed on memory T cells (3, 7, 12, 13). We now report the generation of A6, a mAb with a very high affinity for the p180 LCA molecule, which binds an epitope very close to the UCHL1 epitope. SDS–PAGE studies of the alloreactive cell clone AMS H10 showed that A6 precipitated a 160- to 170-kDa as well as a 180-kDa molecule. The peptide of 160–170 kDa was similar in MW to the 165-kDa molecule precipitated by UCHL1. This is the same MW reported by other laboratories for UCHL1 (2), although other reports indicate a MW of 180 kDa for this antigen (6). The variation in MW may be related to the degree of glycosylation of LCA isoforms in different cell lines or clones. Preclearing with a CD45 mAb removed both bands showing that they were members of the LCA family. In these studies, immunoprecipitation with A6 also showed coprecipitation of molecules of similar MW to those reported to precipitate with UCHL1 and other LCA mAb. Previous reports have shown that LCA molecules are associated and coprecipitate with other cell surface molecules, including CD2, CD4, and CD8, and a 30-kDa phosphorylated protein (27–30). Bands of molecular weight consistent with these molecules were also precipitated by A6. Immunoprecipitation studies of unstimulated radiolabeled PBMC cell lysates showed that A6, like UCHL1, precipitated a single molecule of 180 kDa, consistent with the lower molecular weight isoform of LCA. No additional bands were detected.

Staining transfected cell lines expressing specific isoforms of CD45 showed expression of the A6 epitope on the CD45RO (180 kDa) isoform. Unlike UCHL1, A6 also weakly stained two other isoforms of LCA: a p190 (using exon B) and a p205 (using exons A and B). This weak staining probably reflects low affinity of the A6 mAb for these isoforms and is consistent with the lack of immunoprecipitation of these isoforms from PBMC-radiolabeled cell lysates. A6 was not expressed on transfectants expressing the p220 (exons A, B, and C) or those including exon C. These studies showed the A6 epitope belonging predominantly to the CD45RO and CD45RB but not RA or RC.

Enzyme sensitivity studies showed that the epitopes recognized by both A6 and UCHL1 are partially dependent on carbohydrates. These results are in accordance with those of Pulido *et al.* (10), who found that the UCHL1 epitope was trypsin and pronase resistant and neuraminidase sensitive, while the opposite was true for the 2H4 epitope. Carbohydrate structures are believed to be the functionally important component of LCA molecules (1), in that mAb CT1 and CT2 directed to carbohydrate structures or murine LCA block CTL-mediated cytolysis (33). A6 did not block CTL which suggests it is not a human homologue for CT1 or CT2.

Staining studies showed that A6 completely blocked UCHL1 binding to PBMC or to a T cell clone which suggests that the epitopes bound by the two mAb are very closely located. A6 consistently stained more cells and with stronger intensity than UCHL1 in both PBL and tissue sections from various organs and this is consistent with the observation that A6 weakly recognizes p190 and p205 LCA isoforms that are expressed by some T cells. The bright population seen in FACS analysis of PBL stained by indirect immunofluorescence with A6 probably represents the memory T cells expressing the p180 isoform also identified by UCHL1 and the intermediate fluorescence population probably represents T cells which express low levels of p180 together with intermediate isoforms of CD45. However the $A6^-$ and $A6^{low}$ populations represent cells expressing the 220-kDa and possibly other intermediate isoforms including a subpopulation of T cells, B cells, and NK cells. A6 stained the majority of TCR-$\alpha\beta^+$ cells and all the TCR-$\gamma\delta^+$ cells, although the proportion of cells staining may vary

15

with age. Our studies are consistent with other reports (35, 36) showing that UCHL1 stains a proportion of T cells with lower intensity than the clearly defined high-staining population. Tissue staining with A6 was similar in distribution to UCHL1 (37) in thymus, lymph node, and spleen, but A6 recognized a greater number of cells than UCHL1. Similar to UCHL1, A6 stains cells in paraffin-fixed sections (31).

A6 stained cells in thymus, lymph node, and spleen, a distribution similar to that described for UCHL1 (10, 37). Staining of tumor cell lines with A6 and UCHL1 was identical with the exception of Jurkat cells which were stained weakly by UCHL1 but strongly by A6. The difference is probably due to the lower p180 expression on this cell line but higher expression of the p190 and/or the p205, consistent with previous reports that Jurkat cells predominantly express an intermediate (200 kDa) isoform of LCA (3, 38).

The high affinity of A6 for the p180 molecule enabled the subdivision of T cells into memory ($A6^{high}$) and naive ($A6^{low}$) cells in a similar way to UCHL1 (8, 13). The $A6^{high}$ cells proliferated maximally to recall antigens and to OKT3, but only moderately to PHA. The complementary $2H4^+$ cells responded strongly to PHA but only weakly to OKT3 and were unresponsive to recall antigens, consistent with previous reports using UCHL1 (8, 12, 13) or anti-LFA-3 (15). In alloimmune responses, PBMC depleted of $A6^{high}$ cells had differing responses to those depleted with UCHL1. Particularly in primary MLC, cells depleted with UCHL1 responded normally (8), while the response after $A6^{high}$ depletion was markedly reduced. Populations depleted of $2H4^+$ cells had normal primary and secondary MLC responses. None of the mAbs tested blocked MLC responses. Depletion of $A6^{high}$ cells from alloreactive T cell populations removed specific cytotoxic T cells, although the mAb alone had no capacity to block cytotoxicity. These studies demonstrate that A6 is a mAb with characteristics similar but not identical to UCHL1 (8, 12, 13). The major functional difference in the subpopulations identified by the two mAbs is that depletion with UCHL1 has little effect on MLC reactivity while depletion with A6 removes alloreactive cells. This suggests that the subpopulation identified by A6 binding $CD45RB^+$ cells may be the principal mediator of alloimmune responses and this possibility was investigated (Berger *et al.*, paper submitted for publication). Briefly, the differences in proliferative responses of $A6^{high}$- and $UCHL1^+$-depleted populations were not due to a greater depletion of $CD4^+$ and $CD3^+$ cells per se, but due to depletion of the population staining with intermediate intensity with A6. Our studies also confirmed that some LCA isoforms associate with other functionally important T cell surface molecules, such as TCR, CD3, CD2, CD4, or CD8, and p30 molecule, whose function remains to be determined. Recent studies show that it is the intermediate isoforms that associate with TCR/CD3 (27–30), and this suggests that cells expressing these isoforms, which can be identified by A6, may be functionally important.

REFERENCES

1. Thomas, M. F., *Annu. Rev. Immunol.* **7**, 339, 1989.
2. Schwiner, R., *In* "Leucocyte Typing IV, White Cell Differentiation Antigens" (W. Knapp, B. Dorken, W. R. Gills, E. P. Rieber, R. E. Schmid, H. Steen, and A. E. G. Kr. Von Der Borne, Eds.), pp. 628–634. Oxford Univ. Press, Oxford, UK, 1989.
3. Smith, S. H., Brown, M. H., Rowe, D., Callard, R. E., and Beverley, P. C. L., *Immunology* **58**, 63, 1986.
4. Streuli, M., Morimoto, C., Schrieber, M., Schlossman, S. F., and Saito, H., *J. Immunol.* **141**, 3910, 1988.
5. Wallace, D. L., and Beverley, P. C. L., *Immunology* **69**, 460, 1990.
6. Terry, L. A., Brown, M. H., and Beverley, P. C. L., *Immunology* **64**, 331, 1988.

7. Akbar, A. N., Terry, L., Timms, A., Beverley, P. C. L., and Janossy, G. J., *Immunology* **140**, 2171, 1988.
8. Morimoto, C., Letvin, N. L., Distaso, J. A., Aldrich, W. R., and Schlossman, S. F., *J. Immunol.* **134**, 1508, 1985.
9. Streuli, M., Matsuyama, T., Morimoto, C., Schlossman, S. F., and Saito, H., *J. Exp. Med.* **166**, 1567, 1987.
10. Pulido, R., Cebrian, M., Acevedo, A., Landazuri, M. O., and Sanchez-Madrid, F. J., *Immunology* **140**, 3851, 1988.
11. Hall, L. R., Streuli, M., Schlossman, S. F., and Saito, H., *J. Immunol.* **141**, 2781, 1988.
12. Merkenschlager, M., Terry, L., Edwards, R., and Beverley, P. C. L., *J. Immunol.* **18**, 1653, 1988.
13. Sanders, M. E., Makgoba, N. W., Sharrow, S. O., Stephany, D., Springer, T. A., Young, H. A., and Shaw, S., *J. Immunol.* **140**, 1401, 1988.
14. Morimoto, C., Letvin, N. L., Boyd, A. W., Hagan, M., Brown, H. M., Kornacki, M. M., and Schlossman, S. F., *J. Immunol.* **134**, 3762, 1985.
15. Sanders, M. E., Makgoba, M. W., June, C. H., Young, H. A., and Show, S., *Eur. J. Immunol.* **19**, 803, 1989.
16. Seong, R. H., Clayberger, C. A., Krensky, A. M., and Parnes, J. R., *J. Exp. Med.* **167**, 288, 1988.
17. Kohler, G., and Milstein, C., *Nature* **256**, 495, 1975.
18. Walker, K. Z., Gibson, J., Axiak, J. M., and Prentice, L. R., *J. Immunol. Methods* **88**, 75, 1986.
19. Moebius, U., *In* "Leucocyte Typing IV, White Cell Differentiation Antigens" (W. Knapp, B. Dorken, W. R. Gills, E. P. Rieber, R. E. Schmid, H. Stein, and A. E. G. Kr. Von Der Borne, Eds.), pp. 314–316. Oxford Univ. Press, Oxford, UK, 1989.
20. Moebius, U., *In* "Leucocyte Typing IV, White Cell Differentiation Antigens" (W. Knapp, B. Dorken, W. R. Gills, E. P. Rieber, R. E. Schmid, H. Steen, and A. E. G. Kr. Von Der Borne, Eds.), pp. 342–343. Oxford Univ. Press, Oxford, UK, 1989.
21. Bayer, E. A., and Wilchek, M., *Methods Biochem. Anal.* **26**, 2, 1980.
22. Hall, B. M., De Saxe, I., and Dorsch, S. E., **36**, 700, 1983.
23. Bishop, G. A., Hall, B. M., Waugh, J., Philips, J., Hovarth, J. S., Duggin, G. G., Johnson, J. R., Sheil, A. G. R., and Tiller, D. J., *Lancet* **2**, 645, 1986.
24. Meuer, S. C., Fitzgerald, K. A., Hussey, R. E., Hodgdon, J. C., Schlossman, S. F., and Reinherz, E. L., *J. Exp. Med.* **157**, 705, 1983.
25. Laemmli, U. K., *Nature* **227**, 680, 1970.
26. Bishop, G. A., and Hall, B. M., *Transplantation* **45**, 967, 1988.
27. Schraven, B., *et al.*, *Nature* **345**, 71, 1990.
28. Dianzan, U., *et al.*, *Eur. J. Immunol.* **22**, 365, 1992.
29. Takeda, A., *et al.*, *J. Biol. Chem.* **267**, 16651, 1992.
30. Berti, E., Aversa, G. G., Soligo, D., Caltoretti, G., Delia, D., Aiello, A., Parravicini, C., and Hall, B. M., *Am. J. Pathol.* **95**, 188, 1991.
31. Schraven, B., *et al.*, *Eur. J. Immunol.* **22**, 1857, 1992.
32. Ralph, S. J., Thomas, M. L., Morton, C. C., and Throwbridge, I. S., *EMBO J.* **6**, 1251, 1987.
33. Le Francois, L., and Beran, M. J., *Nature* **314**, 449, 1985.
34. Rudd, C., Morimoto, C., Wang, L. L., and Schlossman, S. F., *J. Exp. Med.* **166**, 1758, 1987.
35. Bernabeu, C., Carrera, A. C., De Londazieri, M., and Sanchez-Madrid, F., *Eur. J. Immunol.* **17**, 1461, 1987.
36. Hayward, A. R., Lee, J., and Beverley, P. C. L., *Eur. J. Immunol.* **19**, 771, 1989.
37. Pilarski, L. M., Gillitzer, R., Zola, H., Shortman, K., and Scollay, R., *Eur. J. Immunol.* **19**, 589, 1989.
38. Yamaguchi, A., Uede, T., Kokai, Y., Ishii, Y., and Kikuchi, K., *Jpn. J. Cancer Res.* **78**(12), 1378, 1987.

17

Use of Monoclonal Antibodies to Study In Vivo and In Vitro-Activated Lymphocytes

G. Aversa, J.A. Waugh, G.A. Bishop, and B.M. Hall

ACTIVATED T lymphocytes play a central role in allograft rejection, directly mediating cytotoxic effector functions or by amplifying the host's immune response to the graft via the release of lymphokines. During activation, T-cells acquire new molecules or increase the expression of others on their surface. Although the function of some of these proteins, such as the interleukin-2 receptor (IL-2R) has been determined,[1] the role of most of these activation antigens remains to be elucidated. We describe the use of three monoclonal antibodies (mAb) for identifying activation antigens on lymphocytes and to visualize activated T-cells in rejected renal allografts.

MATERIALS AND METHODS

Monoclonal Antibodies

The hybridoma lines secreting A1, A4 and A6 (all IgG1) were derived from three fusions as described.[2] OKT26a (anti-IL2-R) was purchased from Ortho (Raritan, NJ). MUD-1 (anti-*D. discoideum*) and OX-8 (anti-rat CD8) were used as controls.

Mononuclear Cells

Peripheral blood mononuclear cells (PBMC) were obtained and activated as previously described.[2]

Flow Cytometry

Resting or activated PBMC were labeled with mAb followed by a second Ab conjugated to FITC as described[2] and analysed with a FACStar (Becton and Dickinson, Mountain View, CA) cell sorter.

Immunoperoxidase Staining of Tissue Sections

Tissue sections from renal specimens were fixed in acetone at RT, then stained as described.[3]

Radioiodination and SDS-PAGE

PHA-activated (day 6) lymphoblasts were radioiodinated using the standard lactoperoxidase-catalyzed reaction. The cell membranes were solubilised with 0.5% NP 40 and precipitated with Pansorbin (Calbiochem), coated with rabbit-anti mouse Ig, and the mAb. The samples were run on a 10% polyacrylamide gel as described.[4] Autoradiography was performed using Kodak X-AR-5 film.

RESULTS

Cell Reactivity of A1, A4 and A6 in Flow Cytometry

A1 reacted strongly with mitogen and alloactivated T cells, but not with resting T cells.[2] A4 reacted moderately with activated T (44%-74%) and B (30%) cells, but not with resting leukocytes. A6 reactivity with donors T cells showed variability (36%-80%), but it reacted strongly with most of activated T-cells (88%-97%). It also weakly stained monocytes (80%-96%) and granulocytes (55%-99%).

Kinetics of Activation Antigens Expression

Flow cytometric analyses of T-cells activated by PHA showed that the expression of A1 and A4 and the increase in A6 expressions were late compared to that of the IL-2R (which peaked at day 3), peaking at day 8, day 6, and day 8, respectively. The kinetics of expression in MLR were delayed peaking at day 16, 10 and 20, while the IL-2R expression peaked at day 8 of MLR.

Immunoperoxidase Staining of Tissue Sections

Sections from rejected renal allografts showed that A1 and A6 strongly stained the cellular infiltrate, whereas their reactivity in normal kidneys was mostly confined to dendritic-like cells in intertubular areas. A1 also stained vascular endothelium and glomeruli.[2] A4 stained large blastlike cells in rejected kidneys, but its staining was weaker than that observed with A1 and A6. A4 did not stain normal kidneys.

SDS-PAGE

A1 precipitated a peptide of 78 kDa in both reduced and unreduced form from the surface of PHA-activated T-cells. A4 precipitation showed two major bands of 83 and 44 kDa in the unreduced form, but only one band of 44 kDa observed in the reduced gel, indicating that the 83 kDa peptide could be the dimeric form of the 44 kDa protein. A6 precipitated a major diffused band in the 160-200 kDa zone and a minor band of 33 kDa in the unreduced form and a main band of 186 kDa and two minor bands of 44 and 33 kDa in the reduced form.

DISCUSSION

We have described 3 mAb reactive with lymphocyte activation markers. A1 recognizes a unique 78 kDa peptide on activated T-cells distinct from those so far described. The peptide precipitated by A4, suggests some similarities with CD 28 (T90/44) recognized by 9.3.[1] However, this remains to be confirmed. A6 reactivity with a subset of resting

From Stanford University School of Medicine, Stanford, California.

Address reprint requests to B.M. Hall, Stanford University School of Medicine, Stanford, CA 94305.

Supported by grants from NH and MRC Australia.

0041-1345/89/$3.00/+0

18

lymphocytes and the m.w. of the main peptides precipitated by it, indicate that it may recognize components of the CD 45 complex, similar to UCHL-1.[5] The expression of the antigens identified by these mAb in cells infiltrating rejected renal allografts may signify an involvement of these structures in the mechanism of allograft rejection. These reagents may be useful in the diagnosis of allograft rejection and possibly as therapeutic agents in the selective elimination of alloreactive T-cells.

REFERENCES

1. Uchiyama T, Broder S, Waldmann TA: J Immunol 126:1393, 1981
2. Aversa GG, Suranyi MG, Waugh JA, et al: Transpl Proc XX(1):49, 1988
3. Bishop GA, Hall BM, Waugh J, et al: Lancet 2:645, 1986
4. Laemmli UK: Nature: 227:680, 1970
5. Terry LA, Brown MH, Beverley PC: Immunology: 64:311, 1988

37.22

Exposure to Non-Inherited Maternal Antigens affects mouse fetal and neonatal T cell development, in an MHC class I transgenic mouse model

Stephane Maurice Caucheteux[1], Yoshinobu Akiyama[2], Cecile Vernochet[1], Marie-Claude Gendron[1], Annick Guimezanes[3], Gilles Benichou[2], Colette Kanellopoulos-Langevin[1]. [1]J. Monod Institute, CNRS, pl. Jussieu, Paris, 75005 France, [2]Massachusetts General Hospital, Boston, MA, [3]CIML, Marseille, France

During fetal and neonatal life, the immune system is exposed to non-inherited maternal antigens (NIMA) absent from fetal self tissues. We are interested in the consequences of such an exposure on lymphoid cell development and adult immune function. We have analysed the influence of the MHC class I molecule H-2Kb as NIMA, in BM3.3 anti-H-2Kb T cell receptor-transgenic (TcR-Tg) animals from day 16 of gestation to day 3.5 after birth. Control mice included BM3.3 TcR-Tg animals that were not exposed to H-2Kb and animals that expressed H-2Kb as a self MHC antigen inherited from the mother (IMA).

NIMA-exposed TcR-Tg animals had a two-fold reduction in their number of DP and CD8-SP thymocytes during gestation. After birth, an increased percentage of thymic cells, compared to negative controls, was in cycle and had a larger size, suggesting an activated status. Messenger RNA analyses by RT-PCR on whole thymus cells revealed an upregulation of IL-4, IL-10, Fas-L and BcL2. Further analysis of the functional status of such activated T cells and of their maturation in the periphery is in progress. Our results show for the first time that exposure to NIMA significantly affects early T cell development. Current work is in progress to determine the mechanisms of NIMA influence on the progeny's immune system and whether it is dependent upon life *in utero* and/or lactation. Supported by FRM, ARC, MENRT and the NATO Science programme.

37.23

Anti-CD45RO/RB mAb a new tool for tolerance induction

Silvia Gregori[1], Patrizia Mangia[1], Rosa Bacchetta[1], Gregorio Aversa[2], Federica Bottazzoli[1], Josè Carballido[2], Josè Carballido[2], Catia Traversari[3], Ulf Korthauer[2], Maria Grazia Roncarolo[1]. [1]San Raffaele Telethon Institute for Gene Therapy (HSR-TIGET), Milan, Milan 20132 Italy, [2]Novartis Research Institute, Vien, Austria, [3]Molmed S.p.A., Milan, Italy

Lymphocytes simultaneously express multiple CD45 isoforms which are transmembrane protein tyrosine phosphatases. We studied the effect of a monoclonal antibody, A6 mAb, which has a unique specificity for both the CD45RO and CD45RB isoforms. A6 mAb is a potent immunosuppresant that inhibited allogeneic and polyclonal responses. High concentrations of A6 mAb induced apoptosis in $CD4^+CD45RO/RB$ bright cells, which is not activation dependent. A6 mAb also modulated antigen(Ag)-specific $CD8^+$ and $CD4^+$ T-cell responses. $CD8^+$ T cell lines specific for the Flu peptide MP58-66 generated in the presence of the A6 mAb became unresponsive to re-challenge with the same Ag. Both IFN-γ production and cytotoxic activity were suppressed in these T cell lines. The anergized $CD8^+$ T cell lines suppressed IFN-γ production by Flu-specific T cell lines, indicating that they act as T regulatory cells. Similarly, $CD4^+$ T cell lines obtained after repetitive stimulations with TT in the presence of A6 mAb became unresponsive and suppressed the proliferation of TT-specific $CD4^+$ T cell lines. Recent data suggest that A6 mAb modulates the effector function of mature DCs. DCs maturated in the presence of A6 mAb and appropriate stimuli induced the differentiation of a regulatory T cell population, which suppressed the proliferative response of autologous naive $CD4^+$ T cells to alloantigenic mature DC. Taken together, these results suggest that A6 mAb may represent a new immunomodulant agent able to induce tolerance, through specific deletion (apoptosis) of recently activated cells, anergy and induction of T regulatory cells.

37.24

Induction of tolerant allogeneic $CD8^+$ T cells by soluble MHC Class I tetramers depends on MHC/TCR avidity

Robert Malle, Catherine A Siler, Samantha Elliott Kerry, Jeffrey A Frelinger. Microbiology and Immunology, University of North Carolina at Chapel Hill, CB #7290, Manning Drive, Chapel Hill, NC 27599

We have previously reported that female C57BL/6 mice injected with three doses of soluble HY (male antigenic peptide)-D^b MHC Class I tetramer rejected male skin grafts more slowly than naïve mice. Using a female HYTCR $RAG^{-/-}$ transgenic mice model we found that the initial phase of tolerance induction resulted in a non-proliferative population of allospecific $CD3^+TCR^+CD4^-CD8^{lo}$T cells ($CD8^{lo}$TC) which correlated with general non-responsiveness to HY antigen. $CD8^{lo}$TC are able to mediate dose-dependent inhibition of naïve HY $CD8^+$ T cell proliferative responses to HY antigen *in vitro*. In order to investigate the role of MHC/TCR affinity in tolerance induction, we have defined weak-agonist (K1A) and super-agonist (C2A) altered peptide ligands (APL) of HY in proliferation and cytolytic assays. We have measured the affinity (K_D) and dissociation constants ($T_{1/2}$) for wildtype (K_D≈200nM, $T_{1/2}$≈60min), C2A (K_D≈200nM, $T_{1/2}$≈110min) and K1A (K_D≈1000nM, $T_{1/2}$≈60min)-tetramers for HYTCR. Three injections of C2A tetramer into HYTCR $RAG^{-/-}$ mice is superior in inducing $CD8^+$ T cell activation, but induce lower numbers of inhibitory $CD8^{lo}$TC (5% of CD8+ T cells) than wildtype HY-tetramer (15%). Interestingly, K1A-D^b does not yield measurable $CD8^{lo}$TC. Interestingly, three doses of C2A-D^b pMHC induces a highly responsive $CD8^+CD44^{hi}$ $CD62L^+CD25^-$ $CD69^-$ memory population. These data allow us to correlate the contribution of initial antigen avidity to the generation of allospecific tolerance.

37.25

Structural and functional heterogeneity in the CD200R family of immunoregulatory molecules

Lydia Lee[1], Zhiqi Chen[1], Simon Wong[2], Gary YuKai[1], Philip A Marsden[2], Reginald M Gorczynski[1]. [1]Transplant Division, Toronto General Hospital, 200 Elizabeth St, Toronto, On M5G 2C4 Canada, [2]Departments of Surgery & Immunology, University of Toronto, Toronto, Ontario Canada

We have previously reported that engagement of CD200-CD200R triggers immunoregulatory signals in several different rodent models, including an allotransplant model and one measuring autoimmune arthritis. We hypothesized that intracellular signaling was mediated mainly by motifs in the cytoplasmic domain of CD200R. We have identified four isoforms of CD200R (R1 to R4), in the mouse gene bank. Our current goal was to characterize structurally these CD200Rs, and examine their expression in different cells and tissues. We have produced heterologous (rabbit) and rat mAbs to the different isoforms following synthesis of peptides conforming to unique sequences in the CD200Rs, and immunizing animals with KLH-peptides. Antibodies were screened for specificity using CHO cells transformed with cDNAs encoding the various isoforms. Thereafter, single cell suspensions from different tissues (spleen, thymus, bone marrow and blood) were screened by indirect FACS analysis. Expression data was confirmed by Western blotting. Distinct tissue distributions were observed for CD200R1/R4 and CD200R2/3. Using the antibodies as blocking reagents, preliminary functional characterization of the different CD200R isoforms confirmed that CD200R1 delivered an immunosuppressive signal in mouse MLCs. The full implications of the tissue-restricted expression of (functionally unique) CD200R isoforms remains to be determined.

Supported by grant from the CIHR (#MT14678) to RMG

37.26

An intact host MHC class I pathway is necessary for long-term islet allograft survival

Joshua Beilke[1], Zachary Johnson[1], Ronald G. Gill[2]. [1]Immunology, Univeristy of Colrado Health Sciences Center, 4200 E. 9th Aveune, Box B-140, Denver, CO 80262, [2]Medicine and Immunology, Univeristy of Colrado Health Sciences Center, Denver, CO

The purpose of this study was to determine the role for CD8 T cells versus generalized MHC class I-restricted antigen presentation in islet

PERGAMON

Molecular Immunology 37 (2000) 107–113

Molecular Immunology

www.elsevier.com/locate/molimm

Effect of somatic hypermutation on potential *N*-glycosylation sites in human immunoglobulin heavy chain variable regions

Deborah Dunn-Walters, Laurent Boursier, Jo Spencer*

Department of Histopathology, GKT Medical School, St Thomas' Campus, Lambeth Palace Road, London SE1 7EH, UK

Received 21 February 2000; accepted 27 March 2000

Abstract

Immunoglobulins are known to be variably glycosylated. Although most carbohydrate is likely to be associated with the constant region, the variable region may also be glycosylated. Variable region glycosylation is known to be associated with antigen binding; in one model, the presence of carbohydrate on the external surface of CDR2 was shown to increase the affinity by up to ten-fold. In this study we have studied the effect of somatic hypermutation on potential sites of *N*-glycosylation in IgV_H genes used by mucosal plasma cells secreting IgM, IgA and IgG in adults and children. Mucosal plasma cells secreting IgM, IgA and IgG are all heavily mutated from childhood. We have observed a tendency to lose the germline encoded *N*-glycosylation sites in all populations studied (a range of 43–67% of genes showing loss of the site). The tendency to lose the site was associated with a higher frequency of somatic hypermutation. We have also analysed the tendency to create potential *N*-glycosylation sites, and observed that this was greater in IgA and IgG than IgM and was again associated with the high frequency of somatic hypermutation. We observed no evidence of selection for either loss or gain of potential *N*-glycosylation sites. The changes in potential glycosylation status associated with a high frequency of somatic hypermutation is likely to increase the diversity of mucosal immunoglobulins, but is not as likely to affect the peripheral immune system where the frequency of somatic hypermutation is generally lower.

Keywords: Immunoglobulin; Hypermutation; Glycosylation; Intestine; Plasma cells

1. Introduction

Immunoglobulins are glycoproteins which are known to vary in their carbohydrate content. Although most analyses of the degree of immunoglobulin glycosylation in health and disease are concerned with constant region glycosylation, it is known that the variable region may also be glycosylated (Wright and Morrison, 1993; Mattu et al., 1998). A minority of human variable regions genes have a potential *N*-glycosylation site (Asn-X-Ser/Thr, where X is any amino acid except Pro, Asp or Glu) in CDR2 or FR3 (Tomlinson et al., 1992). The actions of glycosidases and glycosyl transferases are not clearly understood, and the factors which control whether a potential glycosylation site is used are not known. However, in some cases it has been possible to demonstrate that these sites are functional and that the carbohydrate added is exposed to potential antigen (Wallick et al., 1988; Wright et al., 1991).

Variable region glycosylation is known to affect antigen binding in various systems. One of the most thoroughly investigated is the binding of α(1-6) dextran, where the presence of carbohydrate on the external surface of CDR2 increases binding by up to ten-fold (Wright et al., 1991). It is thought that this could be due to the additional effect of hydrophilic carbohydrate/carbohydrate interaction between antibody and antigen. It is important to determine whether there is potential variability in the degree of glycosyla-

* Corresponding author. Tel.: +44-207-928-9292 ext. 3562; fax: +44-207-401-3661.

E-mail address: jo.spencer@kcl.ac.uk (J. Spencer).

0161-5890/00/$ - see front matter
PII: S0161-5890(00)00038-9

21

tion generated during the life of plasma cell precursors, and whether this might be subjected to selective pressure.

Somatic hypermutation introduces predominantly single nucleotide substitutions into immunoglobulin variable region genes (Jacob et al., 1991). There are hotspots which are frequently mutated and coldspots which are rarely mutated, but most bases fall into neither of these categories, being mutated occasionally (Yelamos et al., 1995). The most commonly mutated G and C nucleotides are generally located in second and third positions, respectively, within 4mer sequences (Dunn-Walters et al., 1998), originally observed as RGYW motifs (where R = A/G, T = C/T and W = A/T) (Rogozin and Kolchanov, 1992). The flanking sequence around the A and T nucleotides which are preferentially mutated is not so clear. The role of somatic hypermutation is thought to be to increase immunoglobulin variability, following activation of B cells by T cell dependent antigens. The rescue of high affinity variants from apoptotic death is thought to be the basis for affinity maturation of the humoral response (MacLennan, 1994).

Human intestinal plasma cells are the effector cells of the mucosal humoral response. The genes encoding the variable regions of mucosal IgM, IgA, and IgG are very highly mutated from childhood, though the basis for this is not known (Dunn-Walters et al., 1997; Boursier et al., 1999). In this study we have analysed the frequency with which potential *N*-glycosylation sites in immunoglobulin variable regions are lost, or gained, in the intestinal plasma cell population as a result of somatic hypermutation. We have focused predominantly on the plasma cells expressing IgV_H4-34 because this is a relatively non-polymorphic gene which can be studied without the additional concern of base changes resulting from polymorphism rather than somatic hypermutation, and also because it has an Asn-His-Ser potential *N*-glycosylation site in CDR2 (codons 52–54).

2. Materials and methods

2.1. Sequences analysed

Expressed, in-frame, sequences analysed were all generated by RT-PCR using RNA isolated from snap frozen human tissues. Normal jejunal biopsies were obtained from five children aged 4.2, 5.2, 5.5, 7, and 13.5 years, respectively. Normal jejunal biopsies from five adults and normal parotid salivary gland from two adults were also studied. Since the majority of cells of B lineage in these specimens are likely to be plasma cells, and since plasma cells contain more RNA than small B cells, the sequences studied are almost certainly those used by plasma cell populations. Different isotypes were studied using a semi-nested PCR method using the following constant region-specific primers; Cμ (5′-CAGGAGACGAGGGGGAA-3′), Cα (5′-GGAAGAAGCCCTGGACCAGGC-3′) and Cγ (5′-CACCGTCACCGGTTCGG-3′) (Boursier et al., 1999; Dunn-Walters et al., 2000). Variable region primers were used as described previously (Dunn-Walters et al., 1997; Boursier et al., 1999).

Genes which are known to be relatively non-polymorphic were studied. Since a number of sequences were studied from each patient, it was possible to observe that not all sequences shared the same base change. We are therefore confident that these base changes were a consequence of somatic hypermutation and did not represent genetic polymorphism.

Genbank accession numbers for this group of sequences are AJ253020–23, 25–33, 35–38, 41, 43–50, 53, 57, 59–63, 66–70, 72–75, AJ222870–76, 80–83, 85–88, 90–93, 95, 96, 98, 99, AJ240570, 72, 73, 78, 80–82, 86, 88–92, 94, 95, AJ240600, and AJ276982–88.

Unused sequences of IgV_H4-34 used for analysis of the distribution of mutations in the absence of selection were out-of-frame between V_H and J_H and were obtained from analyses of DNA. The DNA was obtained by microdissection from immunohistochemically stained tissue sections of human intestine. This enabled analysis of DNA isolated specifically from the plasma cell population (Dunn-Walters et al., 1997). Genbank accession numbers for these sequences are: X87032, Y13169, 72 Y16646–9, Z738020–21, Z80389, Z80708, Z93132, 34, 38, 41, 53–54, 56, 58–59.

2.2. Statistical methods

The number of mutations within groups of genes was compared using Student's *T*-test because the populations were normally distributed. Differences in the tendency for different populations to retain or create potential *N*-glycosylation sites were compared using χ^2.

3. Results

3.1. Loss of the Asn-X-Ser/Thr site (codons 52–54) in CDR2 of IgV_H4-34

The DNA sequence in codons 52–54 of IgV_H4-34 (Fig. 1) was analysed in μ and α heavy chains from adult and paediatric intestinal plasma cells and adult salivary gland plasma cells. In addition, γ heavy chains were analysed in the adult lamina propria plasma cell population. We observed that in most groups, more than half of the sequences had lost this potential *N*-glycosylation site and that there was no significant

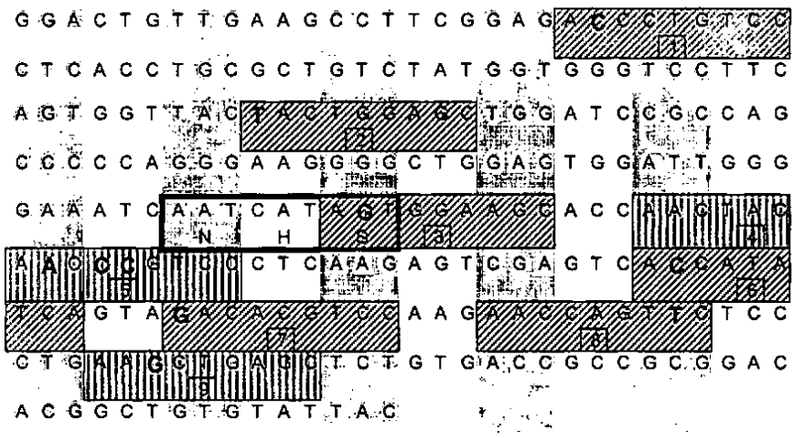


Fig. 1. Sequence of IgV_H4-34 illustrating the location of sites, numbered 1–9 which could become glycosylated as a consequence of a single replacement mutation. The bases which would need to be replaced to produce an *N*-glycosylation site are in bold type. Sites 1–9, respectively, which are all hatched, are codons 17–19, 33–35, 54–56, 58–60, 60–62, 68–70, 72–74, 76–78 and 81–82a. Potential sites of glycosylation which were created most frequently are hatched vertically. The germline encoded NHS site at codons 52–54 is boxed but unshaded.

difference between the groups studied (Table 1). In a group of eight IgV_H32 genes (seven IgA, one IgM) which also have an *N*-glycosylation site encoded in the germline (codons 58–60; Tomlinson et al., 1992), seven had lost this site as a consequence of somatic hypermutation. When all used sequences in the study were divided into those which had lost the *N*-glycosylation site and those which had not, there was a significant difference in the frequency of somatic hypermutation over the entire V region studied within the two groups. The mean frequency of somatic hypermutation in the sequences which had lost the *N*-glycosylation site was 10.1 compared to 6.0 in the sequences which had not lost the site ($p < 2 \times 10^{-8}$ using Student's *T*-test; both groups were normally distributed) (Fig. 2).

We analysed the total number of mutations which occurred in this potential *N*-glycosylation site in the group of genes studied and in a set of out-of-frame genes which are mutated, but which are not expressed and therefore can be considered to be unselected. We

Table 1
Number of IgV_H4-34 sequences used by plasma cells from salivary gland or lamina propria which lost or gained potential *N*-glycosylations sites due to the effect of somatic hypermutation[a]

	Number of sequences at which the N-H-S potential glycosylation site is lost/total	Number of sequences in which one new potential glycosylation site was generated/total	Number of sequences in which two new potential glycosylation sites were generated/total
Paediatric gut IgM	6/10 (60%)	0/10	0/10
Paediatric gut IgA	7/13 (53%)	4/13 (31%)	1/13 (8%)
Adult gut IgM	14/25 (56%)	2/25 (8%)	0/25
Adult gut IgA	17/26 (56%)	7/26 (27%)	0/26
Adult gut IgG	7/16 (43%)	4/16 (25)#	1/16 (6%)
Adult salivary IgM	5/11 (45%)	0/11	0/11
Adult salivary IgA	10/15 (67%)	2/15 (13%)	0/15
Total IgM	25/46 (54%)	2/46 (4.3%)#*	0/46
Total IgA	34/54 (63%)	13/54 (24%)*	1/54 (2%)

[a] Figures marked # ($p = 0.016$) and those marked * ($p = 0.003$) were significantly different when compared using χ^2 analysis.

observed that there was no single hotspot and no apparently selected mutation likely to be responsible for loss of the *N*-glycosylation site (Fig. 3).

3.2. Creation of new potential N-glycosylation sites

N-Glycosylation sites were created by somatic hypermutation in 19 of the total of 116 sequences studied (Table 1). Although there was no significant difference between the tendency to create *N*-glycosylation sites in individual groups of IgA and IgM sequences, there was a significantly greater tendency for these sites to be created in IgA than in IgM, when the groups were pooled ($p = 0.003$ using χ^2). There was also a greater tendency for these to be be created in IgG than in IgM ($p = 0.016$ using χ^2). When the sequences were divided into those which had gained an *N*-glycosylation site and those which had not, the group which had a new potential *N*-glycosylation site was significantly more highly mutated overall (mean frequency of mutation = 9.4%) than the group which had not ($p = 0.00075$ using Student's *T*-test; both groups were normally distributed) (Fig. 2). There was no difference between the frequency of mutation in the groups which had created and lost the potential *N*-glycosylation site.

Two new potential *N*-glycosylation sites were created in each of the two sequences. These had lost the germline encoded glycosylation site and therefore had a total of two sites. Only four sequences which had gained a new glycosylation site had retained the glycosylation site encoded in the germline. Therefore, out of the total 116 sequences studied, only six had two potential sites for *N*-glycosylation, and none had more.

No new potential *N*-glycosylation sites were created in a group of 26 IgV_H5-51 genes used by intestinal plasma cells. This group had a mean frequency of mutation of 5.2%. The frequency of mutation within this population was not significantly different to that of the

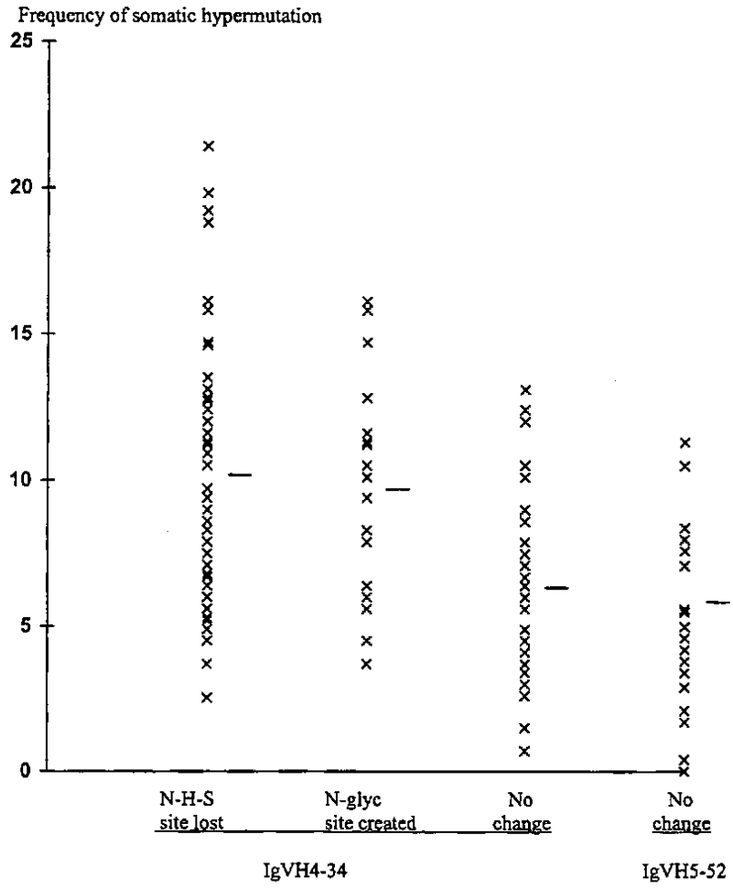


Fig. 2. Frequency of somatic hypermutation in groups of IgV_H4-34 sequences which gained and lost sites of potential *N*-glycosylation, and groups of IgV_H4-34 and IgV_H5-51 sequences which remained unchanged in terms of sites of potential V region glycosylation. The sequences which lost the N-H-S potential site of *N*-glycosylation and the sequences which gained a site had a significantly higher frequency of somatic hypermutation than the sequences which had no change. All in-frame genes derived from analysis of all isotypes are pooled in this figure.

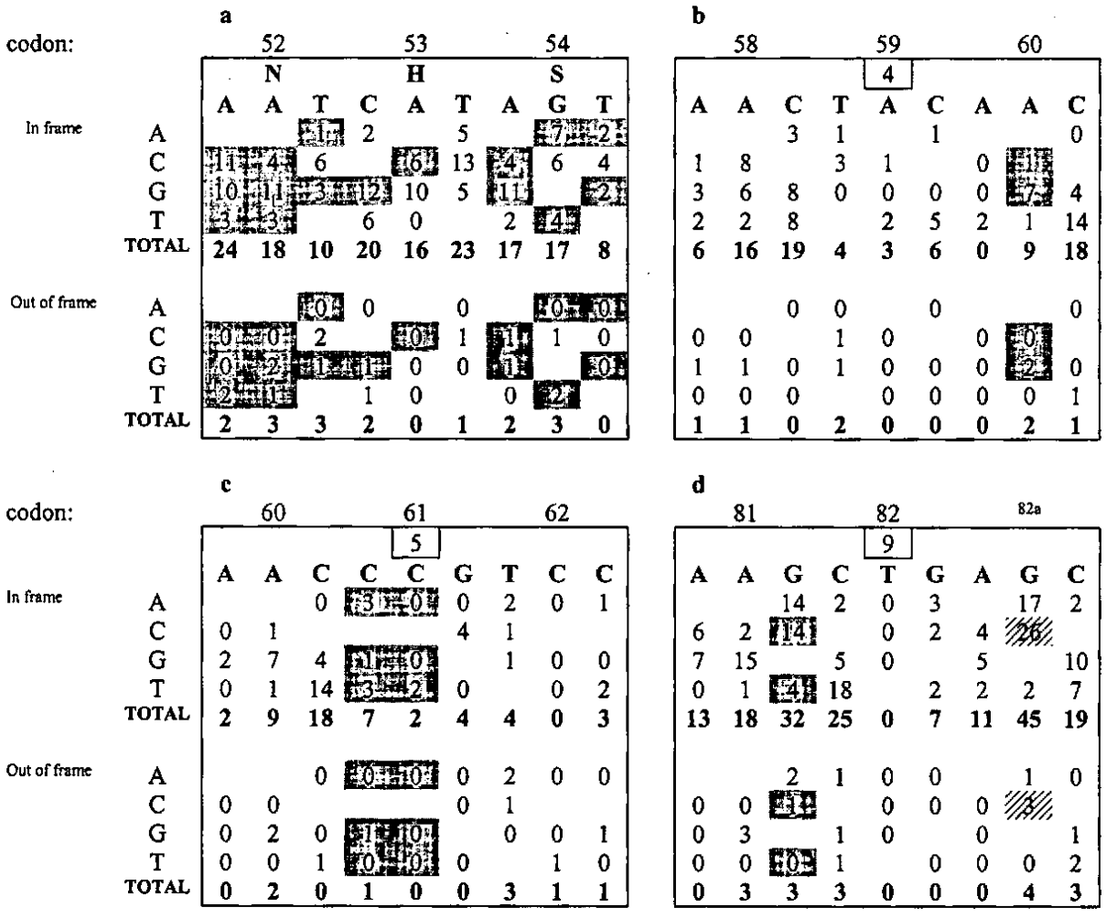


Fig. 3. Analysis of the events at the germline encoded potential *N*-glycosylation site in IgV_H4-34 at codons 52–54 and at the sites 4, 5 and 9 identified in Fig. 1 at codon 58–60, 60–62 and 81–82a. The number of events in used (in-frame) and unused (out-of-frame) alleles have been compared. A total of 1826 mutations in the entire V region were in the used data set, and 245 mutations in the unused data set. Base changes which would result in a change in the potential glycosylation status of the gene are shaded in grey. An interesting hotspot in codon 82a, at which a conservative change can be observed in both the in-frame and out-of-frame alleles, is hatched. There is no evidence of selection either for or against the generation of sites of potential *N*-glycosylation.

IgV_H4-34 genes which did not have *N*-glycosylation sites either created or destroyed (Fig. 2).

3.3. Location of N-glycosylation sites created by somatic hypermutation

We identified nine places in the IgV_H4-34 sequence where a single base change would result in the creation of a potential *N*-glycosylation site which we numbered 1–9 (Fig. 1). Of these nine places, potential *N*-glycosylation sites were only ever observed in six. The majority occurred at three sites only (Fig. 1, Table 2). To determine whether these represent hotspots for somatic hypermutation and to identify any evidence of selective pressure we analysed the frequency of mutation in the relevant codons in the sequences used for this study and a group of out-of-frame sequences which are mutated, but unselected for comparison (Fig. 3). The potential glycosylation sites generated at sites 4 and 5 were not hotspots for mutation and there was no evidence of selective bias in the distribution of mutations.

Table 2
Location of codons which become sites of potential *N*-glycosylation as a result of somatic hypermutation in 19 gut and two salivary gland sequences

Potential created *N*-gly site	Gut	Salivary gland
3[a], codons 54–56	0	1/2
4, codons 58–62	6/19	1/2
5, codons 60–61	6/19	0
6, codons 68–70	1/19	0
9, codons 81–82a	6/19	0

[a] Number of site in Fig. 1.

Site 9 was of interest. The Gs in codons 81 and 82a are both hotspots for mutation, and are both in RGYW motifs. The G in codon 81 is that which results in the creation of the *N*-glycosylation site. Since this is also mutated in the out-of-frame alleles, it is likely to be due to targeting rather than selection. The hotspot at codon 82a is the most highly mutated base in IgV_H4-34 in this series of sequences. It is interesting to note that the most common base change is a transversion from G to C (G most commonly mutates to A; Yelamos et al., 1995). This trend is apparent in both the in-frame and the out-of-frame data. This substitution would result in an amino acid change from serine to threonine, a conservative change in terms of any potential *N*-glycosylation sites generated by changes at codon 81.

4. Discussion

It is clear from this analysis that a high frequency of somatic hypermutation, as observed in the human intestinal plasma cell population, can destroy and create potential sites of *N*-glycosylation in immunoglobulin variable regions. The loss and gain of potential glycosylation sites does not appear to be specifically targeted by somatic hypermutation, but to be a consequence of changes in bases which are neither hotspots nor coldspots. There are sites in the sequence designated '9' in Figs. 1 and 3 which are hotspots for somatic hypermutation. However, the overall effect on potential glycosylation is no different to that in sites 4 and 5, where no hotspot is apparent. This suggests that the changes which occur at high frequency in sequence 9, which may have generated a potential *N*-glycoslation site, are neutralised by changes in the flanking sequence. Since the loss of potential sites of *N*-glycosylation appears to be related to the high frequency of somatic hypermutation and not to targeting of hotspots, we would predict that the peripheral repertoire, where the frequency of somatic hypermutation is much lower, would not show similar changes in potential *N*-glycosylation. Consistent with this, only 20% of IgV_H4-34 genes used by IgM plasma cells from the spleen, which are mutated with a lower frequency (2.3% in 13 sequences), had lost the Asn-X-Ser/Thr site.

Intestinal IgM, IgA and IgG all tended to lose the potential *N*-glycosylation site. In the intestine, IgM, IgA, and IgG are all highly mutated from childhood, though IgA and IgG are significantly more highly mutated than IgM. It appears paradoxical then that, despite the association between the frequency of somatic hypermutation and loss of the germline encoded *N*-glycosylation site, there is no difference between the isotypes in the tendency to lose this site. However, there is a range in frequency of somatic hypermutation within each isotype and the data in Fig. 2 represent a tendency for the *N*-glycosylation site to be lost in the more highly mutated sequences within the individual groups. There was no such paradox when the tendency to create sites was analysed; IgA and IgG had by far the greatest tendency to create sites. This is, again, likely to be due to the higher frequency of somatic hypermutation associated with these isotypes.

We have considered whether the potential changes in variable region glycosylation may have been selected, by comparison of the data with a set of out-of-frame genes, which being unused alleles are mutated, but not subjected to selective pressure. A comparatively small number of sequences was available for the database of unselected genes. However, the relative frequency and distribution of mutations in this set of sequences suggests that the potential changes in glycosylation are not a consequence of selection since the same trends are apparent in the in-frame and out-of-frame genes.

Although somatic hypermutation can generate and remove sites of potential *N*-glycosylation, the strongest overall trend is to remove germline encoded potential sites of *N*-glycosylation. These are present in a minority of variable region genes, including IgV_H4-34 which is thought to represent approximately 10% of the total repertoire. Somatic hypermutation removes the potential N-glycosylation site in over 40% of these genes when they are used by intestinal plasma cells. This is likely to broaden the antibody repertoire since the presence or absence of carbohydrate in CDR2 is known to affect antigen binding (Wallick et al., 1992; Wright et al., 1991; Coloma et al., 1999). It is difficult to understand the biological significance of this potential change in the carbohydrate content, however. In a model investigating the effect of CDR2 linked carbohydrate on binding to α(1-6) dextran, the presence of carbohydrate was reported to enhance binding, and it was considered possible that this may be due to carbohydrate interactions between antigen and antibody (Wright et al., 1991). By the same logic, it might be expected that the presence of carbohydrate in CDR2 may enhance binding to other antigens rich in carbohydrate, such as bacterial wall components. It is difficult to understand why such potentially enhanced binding should be lost from the antibodies secreted by intestinal plasma cells. Similarly, it is possible that glycosylation of IgA may protect IgA from attack by proteases in the intestinal lumen. However, this is not a factor which could be selected for or against in the germinal centre microenvironment.

The question of whether intestinal antibodies are selected for specificity is not fully answered (Dunn-Walters and Spencer, 1998). Intestinal antibodies are

encoded by genes which have some hallmarks of selection, such as an excess of silent mutations in the framework regions compared to out-of-frame, unselected alleles. However, selection for functional viability by conservation of the framework regions and selection for antigenic specificity may be different issues. The question of whether there is selection for efficient antigen binding of intestinal immunoglobulin generally, to which the change in affinity caused by carbohydrate content could contribute, is unanswered at present.

The difference in the characteristics of the IgV_H5-51 and the IgV_H4-34 genes used by intestinal plasma cells is of interest. The former has a lower frequency of somatic hypermutation (Boursier et al., 1999), which is equivalent to the frequency of mutation in the IgV_H genes which did not undergo changes in potential glycosylation status. Like IgV_H 4-34, IgV_H5-51 has sites which could result in the generation of potential *N*-glycosylation sites as a consequence of single base changes. It is possible that there is selection against such changes in IgV_H5-51. The V_H5 family is known to be preferentially associated with IgE responses (Snow et al., 1995). The difference in the frequency of mutation and glycosylation may reflect functional differences between the different IgV_H families.

In conclusion we have shown that the high frequency of somatic hypermutation observed in the human gut can potentially change variable region glycosylation by either the loss or gain of N-linked carbohydrate, though the tendency to lose carbohydrate is the greatest. These changes appear not to be specifically targeted, or selected for or against in the intestinal plasma cell population. However, they are likely to contribute significantly to the functional diversity of the intestinal immunoglobulin repertoire.

References

Boursier, L., Dunn-Walters, D.K., Spencer, J., 1999. Characteristics of IgV_H genes used by human intestinal plasma cells from childhood. Immunology 97, 558–564.

Coloma, M.J., Trinh, R.K., Martinez, A.R., Morrison, S.L., 1999. Position effects of variable region carbohydrate on the affinity and in vivo behavior of an anti-(1-6) dextran antibody. J. Immunol. 162, 2162–2170.

Dunn-Walters, D.K., Boursier, L., Spencer, J., 1997. Hypermutation, diversity and dissemination of human intestinal lamina propria plasma cells. Eur. J. Immunol. 27, 2959–2964.

Dunn-Walters, D.K., Dogan, A., Boursier, L., MacDonald, C.M., Spencer, J., 1998. Base-specific sequences that bias somatic hypermutation deduced by analysis of out-of-frame human IgV_H genes. J. Immunol. 160, 2360–2364.

Dunn-Walters, D.K., Hackett, M., Boursier, L., Ciclitira, P.J., Morgan, P., Challacombe, S.J., Spencer, J., 2000. Characteristics of human IgA and IgM genes used by plasma cells in the salivary gland resemble those used in the duodenum but not those used in the spleen. J. Immunol. 164, 1595–1601.

Dunn-Walters, D.K., Spencer, J., 1998. Strong intrinsic biases towards mutation and conservation of bases in human IgV_H genes during somatic hypermutation prevent statistical analysis of antigen selection. Immunology 95, 339–345.

Jacob, J.G., Kelsoe, G., Rajewsky, K., Weiss, U., 1991. Intraclonal generation of antibody mutants in germinal centres. Nature 354, 389–392.

MacLennan, I.C., 1994. Germinal centres. Ann. Rev. Immunol. 12, 117–139.

Mattu, T.S., Pleass, R.J., Willis, A.C., Kilian, M., Wormald, M.R., Lellouch, A.C., Rudd, J.M., Dwek, R.A., 1998. The glycosylation and structure of human serum IgA1, Fab and Fc regions and the role of *N*-glycosylation of Fca receptor interactions. J. Biol. Chem. 273, 2260–2272.

Rogozin, I.B., Kolchanov, N.A., 1992. Somatic hypermutation of immunoglobulin genes. Part II: Influence of neighbouring base sequences on mutagenesis. Biochim. Biophys. Acta. 1171, 11–18.

Snow, R.E., Chapman, C.J., Frew, A.J., Holgate, S.J., Stevenson, F.K., 1995. Analysis of IgV_H region genes encoding IgE antibodies in splenic B lymphocytes of a patient with asthma. J. Immunol. 154, 5576–5581.

Tomlinson, I.M., Walter, G., Marks, J.D., Llewelyn, M.B., Winter, G., 1992. The repertoire of human germline VH sequences reveals about 50 groups of VH segments with different hypervariable groups. J. Mol. Biol. 227, 776–798.

Wallick, S.C., Kabat, E.A., Morrison, S.L., 1988. Glycosylation of a VH residue of a monoclonal antibody against α(1-6) dextran increases its affinity for antigen. J. Exp. Med. 168, 1099–1109.

Wright, A., Morrison, S.L., 1993. Antibody variable region glycosylation: biochemical and clinical effects. Springer. Semin. Immunopathol. 15, 259–273.

Wright, A., Tao, M., Kabat, E.A., Morrison, S.L., 1991. Antibody variable region glycosylation: position effects on antigen binding and carbohydrate structure. EMBO J. 10, 2717–2723.

Yelamos, J., Klix, N., Goyenechea, B., Lozano, F., Chui, Y.L., Gonzalez, Fernandez, A., Pannell, R., Neuberger, M.S., Milstein, C., 1995. Targeting of non-Ig sequences in place of the V segment by somatic hypermutation. Nature 376, 225–229.

27