Brief Definitive Report

N-LINKED GLYCAN MODIFICATION ON ANTIGEN-PRESENTING CELLS RESTORES AN ALLOSPECIFIC CYTOTOXIC T CELL RESPONSE

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T cells carry an antigen-specific receptor that requires a physical complex of an MHC molecule and a peptide ("processed antigen") derived from the antigen to be recognized, e.g., a viral antigen. Lack of responsiveness can be the result of the absence from the available repertoire of an antigen-specific TCR with a proper fit, or from the inability to properly present the peptide to TCRs. The latter situation could arise because no suitable peptides are generated from a particular antigen, or because the MHC antigen, for intrinsic structural reasons, cannot present the peptide to the antigen-specific receptor. The polymorphic nature of MHC antigens is intimately linked to their ability to present certain peptides: the residues that specify polymorphic variants are located in or in close vicinity of the peptide binding groove (1). Human and most murine MHC class I antigens contain one and two, respectively, N-linked complex carbohydrates that are located at the extremities of this peptide binding groove (1). The possible contribution of these N-linked glycans to recognition of the class I antigen-peptide complex by the TCR remains controversial and is the subject of the present study. It has been reported that the presence of the N-linked glycan is not required for recognition of class I antigens by T lymphocytes (2–4), although it may affect their cell surface expression (5) recognition or accessibility as visualized by antibody binding (6).

An informative model system to study T cell recognition has been the B6 mouse, and spontaneous mutants derived from it. Some of the mutants no longer respond to antigens against which the B6 mouse is a responder (6, 7). In selected cases, the nonresponsive state can be overcome if the antigen is presented by specialized APCs, so-called dendritic cells (DC) (7). DC contain much larger quantities of MHC antigens than do splenocytes; moreover, the amount of sialic acids carried by MHC molecules on DC is significantly reduced when compared with splenocytes, or to other lymphoid cells (6). Stimulation with dendritic cells decreases or obviates the

This work was supported by the Dutch Cancer Society (KWF 85/9) and Medigon (900-509-092).

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$CD4^+$ helper cell requirement in a variety of cytotoxic T lymphocyte responses (7, 8). Splenocytes of bm14 mutant mice, themselves unable to induce an HY-specific CTL response, will acquire this capacity after treatment with neuraminidase (NANAse) (6). Because NANAse treatment fails to discriminate between sialic acids on N-linked glycans, O-linked glycans and glycolipids, experiments were carried out to assess more accurately the role of glycosylation. Here we show, using a B6 anti-bm6 allospecific CTL response, that modifications of a single branch of N-linked glycans can determine whether or not a successful response ensues.

Materials and Methods

Induction and Measurement in T Cell Responses. Responder cells (spleen cells from unprimed C57 BL/6, [B6], mice) were cultured in eight twofold serial dilutions, starting at 10^6 cells, with irradiated (2,500 rad) stimulator cells (LPS-induced B cell blasts from B6. C-H-2^{bm6}, bm6 mice) subjected to treatment with NANAse or cultured in the presence of inhibitors as described below, in 200 μl of culture medium in 96-well round-bottomed microtiter plates.

After 5 d the effector cells were tested on ^{51}Cr-labeled target cells (2×10^3). Cytolytic activity, calculated from individual dose-response curves is expressed as lytic units (LU) per 10^6 responder cells (6). 1 LU is defined as the number of lymphoid cells necessary to obtain half-maximal lysis of the target cells under the assay conditions used.

Treatment of Stimulator Cells. B cell blasts were obtained from spleen cells after culture for 3 d in the presence of 30 μg of LPS/ml (LPS-B, Bacto Laboratories, Detroit, MI). LPS blasts (5×10^7/ml) were treated with neuraminidase for 30 min at 37°C with 2 U of NANAse (*Clostridium Perfringens* type V; Sigma Chemical Co., St. Louis, MO) per milliliter. Inhibitors (1-deoxymannojirimycin [dMM], 1 mM; swainsonine [SW], 3 μg/ml) were added at the beginning of the 3-d culture in the presence of LPS. Before using stimulator cells in the induction of T cell responses, the cells were washed to remove NANAse or inhibitors (11).

Depletion of T Cell Subsets. Purified $CD4^+$ (L3T4) or $CD8^+$ (Lyt-2) T cell subpopulations were obtained through passage of spleen cells over nylon wool and subsequent treatment with either anti-Lyt-2.2 (1:1,000 dilution of ascites fluid; New England Nuclear, Boston, MA) or anti-L3T4 (1:40 dilution of hybridoma culture supernatant SN 172.4, a gift from Dr. H. R. MacDonald, Epalinges, Switzerland) and complement as described (6) and checked for efficacy by cytofluorimetry.

Biochemical Analysis. 5×10^6 bm6 LPS blasts cultured with dMM, SW, or NANAse as described for the MLR, were surface-labeled using lactoperoxidase catalyzed iodination. Cells were lysed in NP-40 containing lysis mix and H-2 antigens were immunoprecipitated using a rabbit polyclonal H-2 serum recognizing H-2K and H-2D locus products, provided by Dr. S. Nathenson, Albert Einstein College of Medicine, New York, NY. Immunoprecipitates were treated with endoglycosidase H where indicated and loaded on a 10% SDS-PAGE or on one-dimensional isoelectric focusing (IEF) (5).

Results and Discussion

Biochemical Analysis. In this study the possible contribution of N-linked glycans to T cell recognition was investigated by applying SW and dMM as inhibitors of N-linked glycan processing mannosidase II and I, respectively. In addition, digestions were carried out with bacterial neuraminidase, a treatment that removes sialic acids from protein bound N- and O-linked glycans as well as glycolipids. Representative structures that result from these treatments are depicted in Fig. 1 *C*. SW produces hybrid-type oligosaccharides, while dMM treatment results in retention of high-mannose type oligosaccharides at positions otherwise occupied by complex type N-linked glycans (5).

Splenocytes from bm6 mice were cultured for 3 d with LPS in the presence or

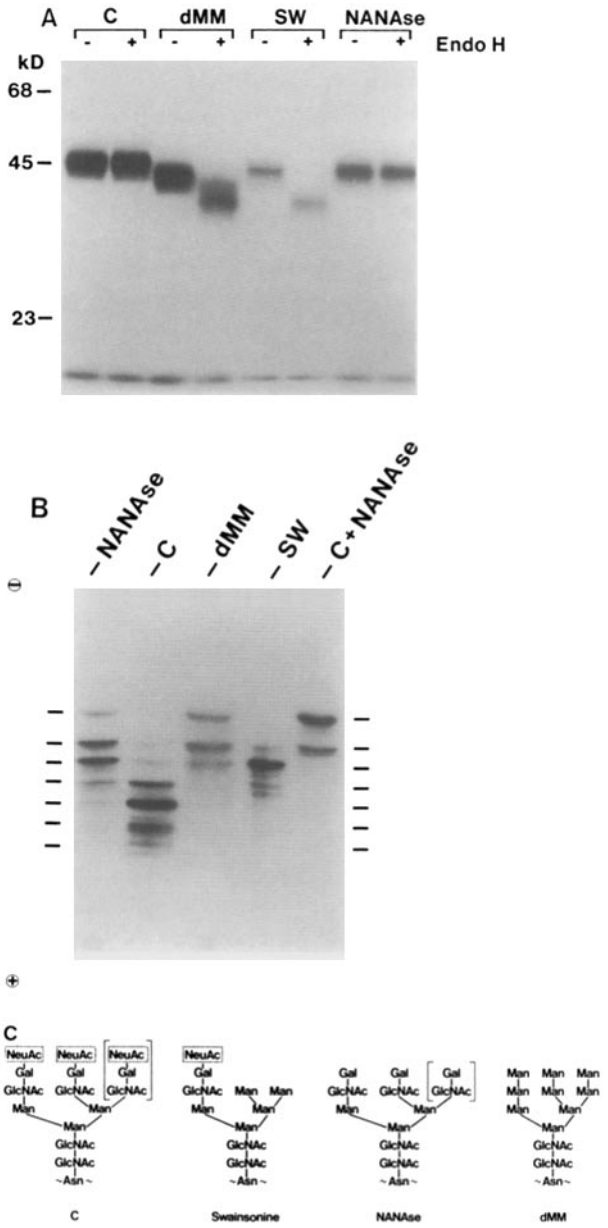

FIGURE 1. Analysis of MHC class I antigens on stimulator cell populations. Bm6 LPS blasts were used as such (C, control), after treatment with NANAse (NANAse), or after a 3-d culture period in the presence of dMM or SW, as indicated. Cells were surface labeled by lactoperoxidase-catalyzed iodination, and H-2 class I antigens immunoprecipitated from them with rabbit anti-mouse class I serum. (*A*) Analysis by SDS-PAGE before and after digestion with endoglycosidase H (Endo H), as indicated. Note that class I antigens from SW- and dMM-treated cells are Endo H sensitive, whereas control and NANAse treated molecules are resistant to Endo H. (*B*) Analysis of class I antigens by one-dimensional IEF. Note the shift in isoelectric point produced upon digestion with NANAse. Digestion of intact cells with NANAse does not result in complete removal of sialic acids, yet the extent of desialylation achieved is more extensive than that obtained with SW. Class I antigens from dMM-treated cells are comparable in their spectrum of isoelectric points to class I molecules from control cells digested with NANAse (lane marked C + NANAse). Class I antigens from SW-treated cells display isoelectric points intermediate to those of control and dMM-treated Class I antigens. The serum used does not discriminate between H-2D^{b} and -K^{bm6} molecules. The lower band of the doublet seen in the C + NANAse lane is H-2K^{bm6} (Ljunggren, H. G., and H. L. Ploegh, unpublished observations). (*C*) Representative structures of N-linked glycans generated in the absence (C, control) or presence of glycosidase inhibitors or NANAse. The NANAse sensitive sialic acids (NeuAc) are boxed.

absence of dMM or SW, or treated with NANAse for 30 min before surface labeling. Class I antigens obtained by immunoprecipitation were analyzed by SDS-PAGE before and after digestion by endoglycosidase H (Fig. 1 *A*). As expected, class I antigens isolated from SW- and dMM-treated cells were sensitive to Endo H. Analysis by one-dimensional IEF was carried out to further characterize class I antigens (Fig. 1 *B*). On control cells, fully sialylated class I heavy chains were present (D^{b} and

K^{bm6} heavy chains show considerable overlap on IEF); in dMM-treated cells virtually complete desialylation was observed. Cultures treated with SW have a hybrid-type oligosaccharide structure where each glycan can contain a single terminally modified glycan and thus a single sialic acid per N-linked glycan. Control cells treated with NANAse yielded class I molecules drastically reduced in sialic acid content, and upon IEF showed a spectrum of isoelectric points very similar to that seen for dMM-treated cells. The common biosynthetic pathway of N-linked glycans assures that all other surface glycoproteins will be modified to an extent similar to that seen for class I molecules. Even though the inhibitors used act almost instantaneously, the half-life of resident glycoproteins will determine the time period at which all surface molecules will have been replaced by glycoproteins with modified glycans. For LPS blasts, 3 d of culture in the presence of glycosidase inhibitors results in a quantitative change of surface class I molecules as determined biochemically.

Effect of Manipulations of N-linked Glycans on APC on the Generation of CTL. In the B6 anti-bm6 primary CTL response, the sole genetic disparity between stimulator and responder animals is a two amino acid difference in the $H\text{-}2K^b$ molecule (6). This antigenic difference results in a weak CTL response that is entirely dependent on the presence of $CD4^+$ cells (Fig. 2) (6).

After depletion of the $CD4^+$ cell population, no response ensues, unless the K^{bm6} antigen is presented on specialized APCs, so called DC (6). The requirement for $CD4^+$ cells with APC other than DC can be dispensed with if exogenous IL-2 is provided (6). We showed previously that DC express larger amounts of class I molecules and their class I molecules carry on average fewer sialic acids (6). Depletion of $CD8^+$ (CTL) cells evidently leads to an inability to mount a CTL response (Fig. 2, hatched bars). Stimulation with LPS blasts fails to evoke a response in the absence of $CD4^+$ cells (Fig. 2; black bars). If the LPS blasts are subjected to NANAse treatment before their use as stimulators, a CTL response of a magnitude comparable to control levels is obtained without the requirement for $CD4^+$ cells (Fig. 2) (6).

The use of LPS blasts maintained in the presence of the inhibitors of glycoprotein processing dMM and SW allowed a further precision of the level at which carbohydrates contribute to the proper evolution of an immune response. When comparing the magnitude of responses generated by unfractionated responder cell suspensions with those of the $CD8^+$ ($CD4^+$-depleted; black bars), the $CD4^+$ ($CD8^+$-depleted;

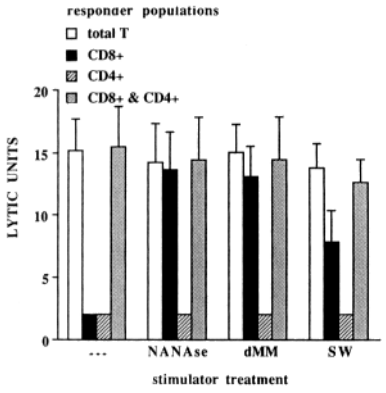

FIGURE 2. Stimulatory capacity of APCs with different carbohydrate structures in the B6 anti-bm6 response. A CTL response was generated in vitro using LPS blasts, NANAse-treated LPS blasts, or LPS blasts cultured in the presence of dMM or SW. CTL assays were then performed with total T cells, $CD8^+$ or $CD4^+$ T cell populations, or a mixture of the $CD4^+$ and $CD8^+$ T cell populations, as indicated in the figure. Lysis was measured on ^{51}Cr-labeled bm6 LPS blasts, and expressed as lytic units. The figure shows mean and standard deviation of four experiments. Using control stimulator cells, the response is entirely $CD4^+$ dependent. Both NANAse treatment and culture of stimulator cells in the presence of dMM results in a full restoration of response, independent of $CD4^+$ cells (black bar). A partial restoration of the B6 anti-bm6 response, independent of $CD4^+$ cells (black bar), is observed when SW-treated LPS blasts are used as stimulator cells.

hatched bars), and the mixture of $CD4^+$ and $CD8^+$ populations (grey bars), the $CD4^+$ cell dependency of the response is observed at a statistically significant level for untreated cells only ($p < 0.001$; Student's t-test). Similar comparisons for NANAse-treated, and dMM- or SW-cultured stimulator cells yielded p-values of >0.1, >0.1, and $0.05 < p < 0.1$, respectively. Thus we conclude that both NANAse and dMM treatment completely abrogate $CD4^+$ cell dependency, whereas SW treatment does so only partially.

Could the effects observed here be explained simply by a reduction of the contribution of sialic acids to the net negative charge of the cells? In this view, elimination of sialic acids would diminish repulsion due to electrostatic interactions. Such charge effects might be expected to be symmetrical, i.e., treatment of responder cells with NANAse would also be expected to result in the generation of a $CD4^+$ cell-independent CTL response. This, however, is not observed (data not shown); only NANAse treatment of stimulator cells, but not of responders, abolishes the $CD4^+$ cell dependence of the B6 anti-bm6 CTL response.

Effects of carbohydrate modifications on antigen presentation have been reported by others also. Examples include treatment of mouse B cells with NANAse, which can stimulate a primary MLR (9); an enhanced stimulation of allogeneic $CD8^+$ cells by NANAse treated macrophages (10); and the restoration of the bm14 anti-HY secondary CTL response by NANAse treatment (6). Powell et al. showed that an allogeneic tumor cell line was recognized after pretreatment with NANAse, or by prior culturing of the tumor cells in the presence of SW (11).

We and others have shown that treatment of cells with NANAse can result in enhanced binding of anti-MHC antibodies as measured by cytofluorimetry (6). These observations show that the accessibility of MHC molecules to the antigen binding sites of antibodies, structurally related to the antigen-binding portions of TCRs, can be improved by removal of the negatively charged sialic acids.

The results reported here are the first that document a biological response that appears to be dependent on the modification of a single antenna of a complex-type N-linked glycan. Subtle changes of carbohydrate structure can occur in vivo, and are observed upon activation of T- and B-cells (decreased sialylation) (9, 12) and malignant transformation (increased sialylation) (13). The effects of dMM and SW are restricted to N-linked glycans only. The K^{bm6} molecule, which carries two such glycans, fails to elicit a response in $CD4^+$-depleted cell populations, if N-linked glycans on the stimulator cell population (including those on K^{bm6} itself) are of the complex type. Possibly the removal of net negative charges from the area of the class I molecule thought to interact with the TCR improves the affinity of this interaction. Alternatively, interactions with accessory molecules might be affected favorably by the absence of sialic acids. At present we can not discriminate between these possiblities. However, the capacity of N-linked glycan modification to modulate lymphocyte responses is clearly established.

Summary

The B6 anti-bm6 allospecific CTL response is strictly dependent on $CD4^+$ cells when using LPS blasts as stimulator cells. Altering the N-linked carbohydrates on stimulator cells by use of the N-linked trimming glycosidase inhibitors 1-deoxymannojirimycin and swainsonine, or by treatment with bacterial neuraminidase,

results in a restoration of the B6 anti-bm6 response in the absence of $CD4^+$ cells. The extent of restoration is inversely correlated with the number of sialic acids present on N-linked glycans of stimulator cells.

Received for publication 2 August 1989 and in revised form 16 October 1989.

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