A red meat-derived glycan promotes inflammation and cancer progression

Annie N. Samraj[a,1], Oliver M. T. Pearce[a,1], Heinz Läubli[a], Alyssa N. Crittenden[a], Anne K. Bergfeld[a], Kalyan Banda[a], Christopher J. Gregg[a], Andrea E. Bingman[a], Patrick Secrest[a], Sandra L. Diaz[a], Nissi M. Varki[a,b], and Ajit Varki[a,c,2]

Departments of [c]Medicine, [b]Pathology, and [a]Cellular and Molecular Medicine, Glycobiology Research and Training Center, University of California, San Diego, La Jolla, CA 92093

Edited by Stuart A. Kornfeld, Washington University School of Medicine, St. Louis, MO, and approved November 25, 2014 (received for review September 18, 2014)

A well known, epidemiologically reproducible risk factor for human carcinomas is the long-term consumption of "red meat" of mammalian origin. Although multiple theories have attempted to explain this human-specific association, none have been conclusively proven. We used an improved method to survey common foods for free and glycosidically bound forms of the nonhuman sialic acid *N*-glycolylneuraminic acid (Neu5Gc), showing that it is highly and selectively enriched in red meat. The bound form of Neu5Gc is bioavailable, undergoing metabolic incorporation into human tissues, despite being a foreign antigen. Interactions of this antigen with circulating anti-Neu5Gc antibodies could potentially incite inflammation. Indeed, when human-like Neu5Gc-deficient mice were fed bioavailable Neu5Gc and challenged with anti-Neu5Gc antibodies, they developed evidence of systemic inflammation. Such mice are already prone to develop occasional tumors of the liver, an organ that can incorporate dietary Neu5Gc. Neu5Gc-deficient mice immunized against Neu5Gc and fed bioavailable Neu5Gc developed a much higher incidence of hepatocellular carcinomas, with evidence of Neu5Gc accumulation. Taken together, our data provide an unusual mechanistic explanation for the epidemiological association between red meat consumption and carcinoma risk. This mechanism might also contribute to other chronic inflammatory processes epidemiologically associated with red meat consumption.

red meat and cancer | *N*-glycolylneuraminic acid | tumor-associated inflammation | tumor-associated carbohydrate antigen | xenosialitis

There is a long-standing epidemiological link between the consumption of red meat (beef, pork, and lamb) and the incidence of carcinomas, atherosclerosis, type 2 diabetes, and all-cause mortality (1–4). Although such diseases have multifactorial origins, all are aggravated by chronic inflammation (5, 6). Red meat-rich diets also correlate with circulating markers of inflammation and endothelial dysfunction (7). Here, we focus on red meat-related risk of carcinomas (further citations regarding the association are provided in Table S1). Corroboration comes from the low rates of carcinomas in populations that consume very low levels or no red meat (8–10). Within the World Cancer Research Foundation report, red meat was among the top 10 factors associated with incidence and progression of carcinomas in all populations (11). Enhancement of carcinoma risk appears to be highest in tissues like colonic epithelium, in which adenomas can progress to carcinomas, driven by molecular changes in oncogenes and tumor suppressor genes (12). There are many proposed mechanisms for the cancer-promoting effects of red meat (13), including generation of mutagens by grilling, DNA damage due to *N*-nitroso compounds, or free radical generation by heme iron. However, none of these mechanisms have been proven, and confounding facts are apparent in some instances (e.g., grilling of poultry and fish generates the same mutagens, yet these foods are not associated with cancer risk) (14). Also, doses of the mutagens that induce carcinomas in animal models are many fold higher than human exposure (15). Overall, although more than one of these theories may still prove to be correct, definitive proof remains lacking.

Another unexplained fact is the human specificity of this risk (i.e., other vertebrate carnivores do not suffer a high incidence of carcinomas). In this regard, we have suggested an unusual human-specific mechanism, involving inflammation associated with metabolic incorporation of a nonhuman sialic acid, *N*-glycolylneuraminic acid (Neu5Gc), and interaction with circulating anti-Neu5Gc antibodies (16–19). Despite the fact that humans are genetically unable to produce Neu5Gc, this molecule is detectable on surfaces of human epithelia and endothelia, and in higher amounts in malignant tissues (20). In the absence of an alternate pathway for Neu5Gc biosynthesis (21), the only possible source for incorporation is dietary intake (22). An initial food survey showed a prominent presence of Neu5Gc in red meat (23). Metabolic incorporation of dietary Neu5Gc into tissues (24) makes this glycan the first example, to our knowledge, of a xeno-autoantigen, which can react with circulating anti-Neu5Gc antibodies (i.e., xeno-autoantibodies) (25). The resulting antigen–antibody interaction is hypothesized to generate or promote chronic inflammation or "xenosialitis," which could contribute to carcinogenesis or to other diseases exacerbated by chronic inflammation.

Significance

We present an unusual mechanism for the well-known association between red meat consumption and carcinoma risk involving the nonhuman sialic acid *N*-glycolylneuraminic acid (Neu5Gc). We first evaluate the Neu5Gc content of various foods to show that red meats are particularly rich in orally bioavailable Neu5Gc and then investigate human-like Neu5Gc-deficient mice fed this form of Neu5Gc. When such mice were challenged with anti-Neu5Gc antibodies, they developed evidence of systemic inflammation. Long-term exposure to this combination resulted in a significantly higher incidence of carcinomas (five-fold increase) and an association with Neu5Gc accumulation in the tumors. Similar mechanisms may contribute to the association of red meat consumption with other diseases, such as atherosclerosis and type 2 diabetes, which are also exacerbated by inflammation.

Author contributions: A.N.S., O.M.T.P., A.N.C., K.B., N.M.V., and A.V. designed research; A.N.S., O.M.T.P., H.L., A.N.C., A.K.B., K.B., C.J.G., A.E.B., P.S., S.L.D., and N.M.V. performed research; A.N.S., O.M.T.P., H.L., and A.K.B. analyzed data; and A.N.S., O.M.T.P., H.L., and A.V. wrote the paper.

Conflict of interest statement: A.V. and N.M.V. are cofounders of and advisors to SiaMab Therapeutics, Inc., which has licensed University of California, San Diego technologies related to anti-Neu5Gc antibodies in cancer.

This article is a PNAS Direct Submission.

[1]A.N.S. and O.M.T.P. contributed equally to this work.

[2]To whom correspondence should be addressed. Email: a1varki@ucsd.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1417508112/-/DCSupplemental.

Although attractive in principle, this hypothesis has not been proven in an in vivo system. Notably, *Cmah*$^{-/-}$ (CMP-Neu5Ac hydroxylase knockout) mice with a human-like deficiency of Neu5Gc (24) can incorporate Neu5Gc of dietary origin, but only if provided in the bioavailable glycosidically bound form (22). However, such mice do not spontaneously generate anti-Neu5Gc antibodies. The likely reason is that humans are spontaneously immunized via an unusual postnatal process involving a human-specific commensal bacterium that presents Neu5Gc from weaning foods to the immune system (26). However, an anti-Neu5Gc response is induced in *Cmah*$^{-/-}$ mice by active immunization, and specific antisera can be generated. Using such antisera in *Cmah*$^{-/-}$ mice bearing syngeneic *Cmah*$^{+/+}$ tumors, we have shown that the anti-Neu5Gc antibodies could facilitate tumor progression by enhancing inflammation (16, 17). However, despite this suggestive evidence, we have not demonstrated that oral feeding of Neu5Gc can induce the proposed xenosialitis in vivo or that this process can increase rates of spontaneous carcinomas. Also, dietary surveys on Neu5Gc in food have been limited, the ratio of bound and free Neu5Gc has not been determined, and effects of food processing have not been considered. Given our hypothesis that tissue incorporation of Neu5Gc of dietary origin can have deleterious effects, it is important to determine the amounts present in typical components of the Western diet accurately. Although earlier studies attempted to quantify Neu5Gc distribution in food (23, 27–29), a systematic catalog of a wide variety of commonly consumed foods is lacking. Furthermore, based on recent studies (22), it appears that glycosidically bound Neu5Gc is the dietary source that is bioavailable for tissue incorporation, and not the free monosaccharide. However, available data neither differentiated between bound and free Neu5Gc nor addressed the effects of food processing.

Here, we address all these issues by first developing an assay to determine amounts of free and bound Neu5Gc accurately in a wide variety of foods. We then evaluate the impact of short-term and long-term feeding of Neu5Gc in *Cmah*$^{-/-}$ mice combined with passive or active immunization against Neu5Gc. Although the association between red meat consumption and colon cancer is most prominent in humans, we focus here on hepatocellular cancer in male C57BL/6 mice as proof of principle, because these animals have a low rate of spontaneously occurring hepatic tumors (30).

Results

Distribution of Free and Bound Neu5Gc and *N*-Acetylneuraminic Acid in Foods. Acid hydrolysis and derivatization by 1,2-diamino-4,5-methylenedioxybenzene dihydrochloride (DMB) followed by RP-HPLC with fluorescent detection (DMB-HPLC) has been the method of choice for the detection and quantification of sialic acids (31). Nevertheless, this method cannot accurately differentiate between bound and free sialic acids, because the conditions of the derivatization reaction (1.5 M acetic acid, 50 °C, 2.5 h) result in some concurrent release of bound sialic acid (this false-positive signal varies with the sample; 8.6% for a model substrate, such as fetuin; Table S2). For the current study, we therefore modified conventional protocols by adapting temperature derivatization conditions (4 °C for 48 h) originally introduced for preserving acid-labile polysialic acid (32). These conditions efficiently tag free sialic acids, without any significant release of bound sialic acids (Table S2). By studying a parallel aliquot with the conventional method, we could accurately quantitate free and bound Neu5Gc. Initial base treatment of samples to eliminate O-acetyl esters also ensured a simplified profile for quantitation.

Using this improved method, we could accurately quantitate bound and free Neu5Gc and *N*-acetylneuraminic acid (Neu5Ac) in foods (examples of HPLC profiles are provided in Fig. 1). As summarized in Table 1 and detailed in Table S3, foods from mammals (cow, goat, sheep, pig, and bison) contain moderate to high amounts of Neu5Gc. Three methods of cooking significantly altered neither Neu5Gc content nor the ratio of bound and free forms. As predicted from prior work (33), poultry and eggs do not contain Neu5Gc and fruits and vegetables do not contain any sialic acids. Although the *CMAH* gene is present in fish, none of the seafood sampled contains significant amounts of Neu5Gc (with the exception of caviar). As shown in Table S3, a great majority of the sialic acids in all samples are glycosidically bound and not free, even after cooking. Overall, the highest levels

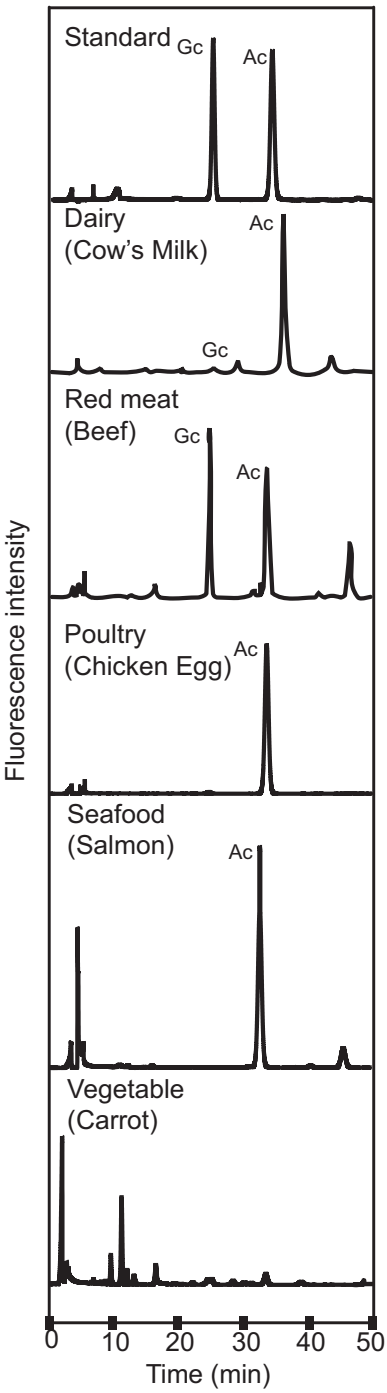


Fig. 1. Examples of DMB-HPLC chromatograms for quantification of Neu5Ac (Ac) and Neu5Gc (Gc) content. Representative examples of results from different food groups are shown. Standard samples were run each day to account for slight variations in peak elution time.

MEDICAL SCIENCES

Table 1. Summary of Neu5Gc content and percentage of Neu5Gc (relative to total sialic acids) of various food groups

Food sample	Neu5Gc content, μg/g	Neu5Gc, %
Dairy		
Butter	0	0
Whole milk	2	12
Cow's milk cheeses	10–22	2–4
Goat's milk cheese	43	44
Red meats		
Bison	29	32
Lamb	14	13
Beef	25–231	32–53
Pork	7–40	9–22
Poultry		
Hen egg	0	0
Turkey	0	0
Chicken	0	0
Seafood		
Fish	0	0
Shellfish	0	0
Caviar	445–530	13–29
Vegetables	0	0
Fruits	0	0

Details within each category of foods are provided in Table S2.

of Neu5Gc among the red meats are in beef, which contains up to 231 μg of Neu5Gc per gram of meat, and the lowest amounts were seen in milk and milk products, with Neu5Gc levels ranging from 2 to 40 μg/g. Beef also contains the highest percent Neu5Gc of total sialic acid, and this Neu5Ac/Neu5Gc ratio may be relevant because of likely competition of Neu5Ac with Neu5Gc for incorporation into cells (34).

Systemic Inflammation Induced in *Cmah*$^{-/-}$ Mice by Dietary Neu5Gc and Circulating Anti-Neu5Gc Antibodies. We have previously shown that anti-Neu5Gc antibodies can influence the growth of transplanted Neu5Gc-positive syngeneic tumors in *Cmah*$^{-/-}$ mice by enhancing chronic inflammation (16, 17). We now asked if oral feeding of Neu5Gc could induce chronic inflammation in vivo, and in a manner dependent on circulating anti-Neu5Gc antibodies. *Cmah*$^{-/-}$ mice were fed a Neu5Gc-free diet or with the same diet supplemented with Neu5Gc-rich porcine submaxillary mucin (PSM), which contains 7–9% (wt/wt) of bioavailable glycosidically bound Neu5Gc, adds only a minimal amount of neutral sugars and amino acids to the diet, and can cause mouse tissue incorporation of Neu5Gc over a period of weeks at levels histologically similar to the levels seen in adult humans who have eaten red meat for many years (22). The question to be addressed is whether the addition of anti-Neu5Gc antibodies to such animals would generate systemic inflammation. Such an anti-Neu5Gc antibody response can be induced by active immunization (26). However, we found that this response is highly variable and difficult to control or manipulate. We therefore challenged Neu5Gc-fed or -nonfed mice by injection of anti-Neu5Gc–rich polyclonal serum, or with a highly specific control serum, prepared as previously described (16, 18).

Cmah$^{-/-}$ mice were fed Neu5Gc-rich or control diets for 12 wk and then injected with varying amounts of control or immune sera calculated to achieve levels of anti-Neu5Gc antibodies in the range found in humans (34). As shown in Fig. 2*A*, evidence of systemic inflammation (elevated levels of peritoneal fluid IL-6 and serum acute-phase proteins, serum amyloid A protein, and haptoglobin) was seen only with the combination of dietary Neu5Gc plus infusion of anti-Neu5Gc antisera, and not with control combinations. Furthermore, the levels of inflammatory markers showed a dose dependency with the amount of antibody injected (Fig. 2*B*). Similar studies were not done in *Cmah*$^{+/+}$ mice, because high levels of endogenous Neu5Gc would neutralize any transferred antibodies (24).

Neu5Gc-Rich Diet Promotes Hepatocellular Cancer Incidence in *Cmah*$^{-/-}$ Mice Only in the Presence of Anti-Neu5Gc Antibodies. To test our hypothesis that oral feeding of Neu5Gc can result in metabolic incorporation and interact with circulating anti-Neu5Gc antibodies, we used the human-like, Neu5Gc-deficient *Cmah*$^{-/-}$ mice bred into a C57BL/6 background, in which occasional male mice develop liver tumors (30). Two groups of such mice were immunized with a Neu5Gc-containing immunogen (porcine RBC ghosts) and fed either a Neu5Gc-rich diet (PSM, containing 0.25 mg of Neu5Gc per gram of chow) or a Neu5Ac-rich diet containing edible bird's nest (EBN; containing 0.25 mg of Neu5Ac per gram of chow). Sera from the two groups showed comparable levels of anti-Neu5Gc antibodies (Fig. 3*A*), and there were no significant abnormalities in hematology or blood chemistry values. Although immunohistological examination of such mice showed Neu5Gc incorporation at levels similar to those levels seen in tissues from red meat-eating humans (22), precise chemical methods for comparing incorporation are not yet available. We therefore monitored serum levels of the inflammatory cytokine IL-6, which is frequently elevated in chronic liver disease and malignancy (35, 36). Significantly higher levels were seen in the *Cmah*$^{-/-}$ mice fed PSM for up to ∼30 wk (Fig. 3*B*). We also monitored the mice with periodic MRI scans. Based on the first appearance of a visible liver lesion by MRI at ∼40 wk (Fig. 3*C*), we chose to necropsy all mice at ∼55 wk. Two of seven *Cmah*$^{-/-}$ mice fed PSM had early hepatocellular carcinomas (HCCs) (Fig. 3*D*), supporting our hypothesis that interaction between anti-Neu5Gc antibodies and Neu5Gc of dietary origin facilitates carcinoma progression.

To confirm and extend the findings, we set up a comparison of *Cmah*$^{-/-}$ and WT mice fed Neu5Gc-rich PSM, and also included control immunizations. We chose human and chimpanzee RBC ghosts as immunogens for this set of experiments because these cell types are very similar, except for Neu5Gc in the chimpanzee. This experimental condition also mimics human populations (25)

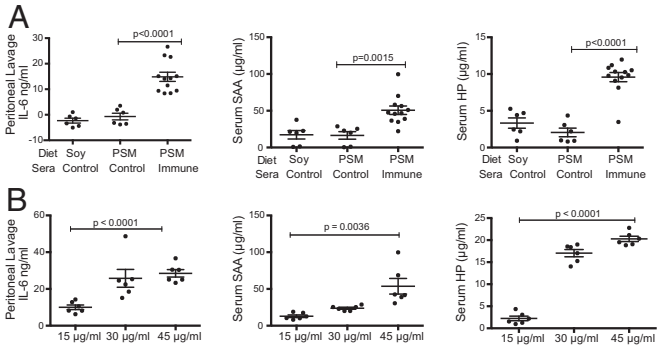
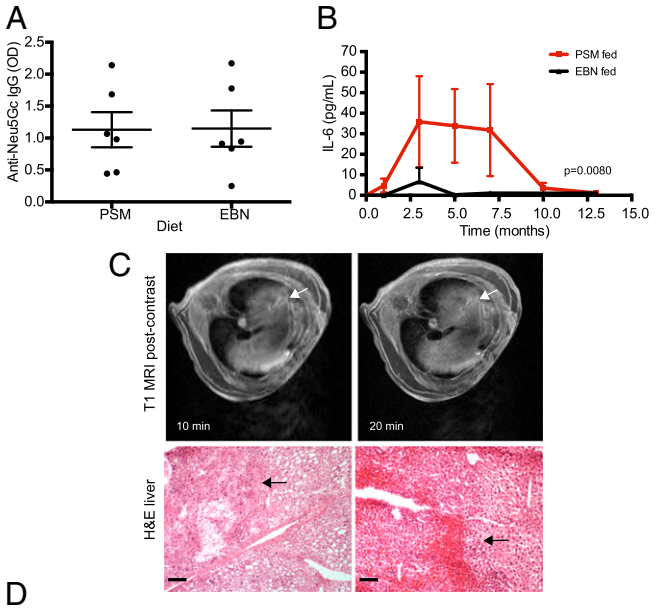


Fig. 2. Dietary Neu5Gc and anti-Neu5Gc antibody-dependent inflammation in Neu5Gc-deficient mice. *Cmah*$^{-/-}$ mice were fed Neu5Gc-free or Neu5Gc-rich diets for 12 wk and then injected with control or anti-Neu5Gc immune sera as described in *Materials and Methods*. Sera and peritoneal lavage fluid were collected after 5 d of transfer of sera and analyzed for various inflammatory markers. (*A*) Inflammatory responses with different combinations of sera and diets. (n = 6 mice in each of the control groups and n = 12 mice in the test group). The probability values are $P < 0.001$, $P = 0.0015$, and $P < 0.001$ for IL-6, serum amyloid A (SAA), and haptoglobin (Hp), respectively. (*B*) Dose dependency of the inflammatory response in Neu5Gc-fed mice injected with immune sera (n = 6 mice in each group). The probability values are $P < 0.001$, $P = 0.0036$, and $P < 0.001$ for IL-6, SAA, and Hp, respectively. Tests of significance were carried out in Prism version 6.0 using the Student t test.



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Genotype	Immunization	Diet	Mice (N)	Adenoma	HCC	HCC%
Cmah $^{-/-}$	Neu5Gc	EBN	6	0	0	0
Cmah $^{-/-}$	Neu5Gc	PSM	7	0	2	29

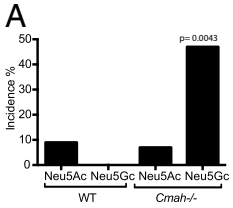
Fig. 3. Spontaneous appearance of HCCs dependent on dietary Neu5Gc and anti-Neu5Gc antibodies. As described in *Materials and Methods*, *Cmah*$^{-/-}$ mice immunized against Neu5Gc were fed a Neu5Gc (PSM)- or Neu5Ac (EBN)-rich diet for a period of ∼55 wk ($n = 7$ in the PSM group and $n = 6$ in the EBN group). (*A*) Anti-Neu5Gc antibody levels in *Cmah*$^{-/-}$ mice at 2 wk post-immunization. (*B*) Significantly higher levels of IL-6 are seen in mice fed a Neu5Gc-rich diet. Tests of significance were carried out in Prism version 6.0 using two-way ANOVA, and the probability value is $P = 0.0080$. (*C*, *Upper*) T1-weighted MRI images postgadolinium injection demonstrating a focal contrast-enhancing lesion in the left lobe of the liver. (*C*, *Lower*) HCC tumor margin (black arrows) in H&E staining of the *Cmah*$^{-/-}$ mouse livers fed a Neu5Gc-rich diet (PSM). (Scale bar: 100 μm; Magnification: ×100.) (*D*) Table detailing genotype, immunization, and HCC rates.

in that the antibody response is polyclonal and variable. We also extended the duration of follow-up to >80 wk, during which variable numbers of mice in each group either died spontaneously or had to be euthanized for severe dermatitis or other moribund states. There was no specific pattern to these losses, and necropsies showed no liver tumors. All surviving mice were finally killed at ∼85 wk. As expected, occasional mice in some groups had hepatic adenomas or HCCs at this late time point (incidence ranging from 0–9%). However, only those *Cmah*$^{-/-}$ mice that were fed PSM and immunized with Neu5Gc-rich RBC ghosts developed HCCs at a substantially higher rate (47%; i.e., the mice expected to have xenosialitis). This experiment was repeated in a second cohort, with even clearer results (i.e., carcinomas occurred only in the *Cmah*$^{-/-}$ mice fed PSM and immunized with Neu5Gc). The raw data from both experiments are shown in Table S4, and the combined data are summarized in Fig. 4*A*. Overall, in striking contrast to all of the other groups, almost half of the *Cmah*$^{-/-}$ mice fed PSM and immunized with chimpanzee RBC ghosts developed HCCs (histological examples are shown in Fig. 4*B*, including an example of lung metastasis). Immunohistochemistry also demonstrated incorporation of Neu5Gc into the tumors (Fig. 4*C*). Taken together, our data show that the combination of dietary Neu5Gc and circulating anti-Neu5Gc antibodies specifically enhances carcinoma rates only in Neu5Gc-deficient animals ($P = 0.0043$), in an organ that is already prone to a baseline rate of tumorigenesis. This observation is very similar to the situation of human red meat eaters, who show an increased incidence of carcinomas in tissues, such as the colonic epithelium, that are already prone to a baseline rate of cancer incidence. To our knowledge, this model is the first example in which incorporation of a dietary molecule combined with antibodies against the molecule is both necessary and sufficient to induce spontaneous carcinomas.

Discussion

We have demonstrated here that antibodies directed against the nonhuman sialic acid Neu5Gc can interact with metabolically incorporated Neu5Gc derived from oral intake and promote inflammation in a dose-dependent manner in the human-like Neu5Gc-deficient *Cmah*$^{-/-}$ mouse model. Furthermore, long-term exposure to such inflammation promotes carcinoma incidence in a target organ where Neu5Gc can accumulate; in the case of mice, it is detected in the liver, and in humans, it is detected more prominently in the colon, prostate, and ovary. Given that glycosidically bound Neu5Gc is relevant for incorporation from dietary sources, we have also quantified free and bound Neu5Gc content in various food products, affirming that foods of mammalian origin are the primary sources.

Chronic inflammation in the context of carcinogenesis and tumor progression has been studied extensively. Although the adaptive immune system can restrict cancer development, it can also paradoxically assist the tumor by promoting chronic inflammation via antibodies directed against tumor cell epitopes (37, 38). Known mechanisms include deposition of sublytic levels of complement with activation of the PI3K/AKT survival pathway (39) and/or Fcγ receptor-dependent polarization of F4/80^{+}/CD11b^{+} tumor-associated macrophages into tumor-promoting M2 phenotypes, thereby suppressing tumor infiltration by activated CD8^{+} lymphocytes (40). Furthermore, it is possible that intratumoral antibody



Genotype	Immunization	Mice (N)	Adenoma	HCC	HCC%
WT	Neu5Ac	11	1	1	9
WT	Neu5Gc	11	0	0	0
Cmah$^{-/-}$	Neu5Ac	14	0	1	7
Cmah$^{-/-}$	Neu5Gc	17	1	8	47

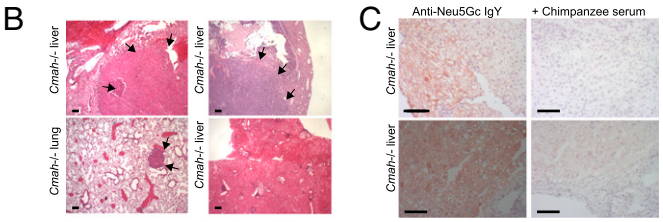


Fig. 4. Neu5Gc-rich diet promotes hepatocellular cancer only in Neu5Gc-deficient mice immunized against Neu5Gc. As described in *Materials and Methods*, WT or *Cmah*$^{-/-}$ mice immunized with Neu5Gc- or Neu5Ac-rich RBC ghosts (chimpanzee or human, respectively) were fed a Neu5Gc-rich (PSM) diet for a period of ∼80–85 wk before necropsy. Combined data from two experiments are shown. (*A*) HCC incidence in four groups of mice is compared: *Cmah*$^{-/-}$ mice immunized with Neu5Gc, *Cmah*$^{-/-}$ mice immunized with Neu5Ac, WT mice immunized with Neu5Gc, and WT mice immunized with Neu5Ac. A markedly higher rate of HCC was seen in the Neu5Gc-fed and Neu5Gc-immunized *Cmah*$^{-/-}$ mice compared with the control groups (the raw data are provided in Table S4). Tests of significance were carried out using the Fisher exact probability test. The two-tailed probability value for the test group (*Cmah*$^{-/-}$ Neu5Gc) compared with the control groups is $P = 0.0043$. (*B*) Representative examples of HCC in *Cmah*$^{-/-}$ mouse liver are shown, with black arrows pointing to the tumor interface. An example of lung metastasis is also shown. (Scale bar: 100 μm; Magnification: ×40.) (*C*) Detection of Neu5Gc in tumors by immunohistochemistry. Representative examples of *Cmah*$^{-/-}$ mouse liver are seen below. Positive staining with anti-Neu5Gc IgY (*Left*) and competitive inhibition with chimpanzee serum as the negative control (*Right*) are shown. (Scale bar: 100 μm; Magnification: × 200.)

MEDICAL SCIENCES

deposition accelerates chronic inflammation by inducing pathways involving COX-2 (41, 42) and NF-κB (43). Release of reactive oxygen species by recruited phagocytic cells could also cause DNA damage, facilitating carcinogenesis (44). On the other hand, there are many dramatic examples of the successful therapy of cancer with monoclonal antibodies (45). In our own recent work, we showed that the range of antitumor antibody levels that bridge the spectrum from tumor activation to inhibition can be remarkably narrow (18).

There are limitations in directly comparing this mouse study and the human situation. Unlike the case in humans, there was a single dietary source of Neu5Gc that was not varied in intake and the antibody production was not sustained throughout the lifetime of the animal. Also, the target organ for the adenoma-to-carcinoma sequence was the liver, not the colon. The obvious question arising from such experimental mouse studies is whether circulating anti-Neu5Gc antibodies correlate with cancer risk in human population studies. We are currently exploring this possibility, but we are also aware of the numerous complicating variables in humans, such as the varying amount and unknown bioavailability of Neu5Gc from dietary red meat, the amount of Neu5Gc tissue loading in benign and malignant tissues, the complex and variable polyclonal antibody profiles of individual humans, the skewed distribution of antibody levels within populations, the possibility that very high antibody levels can inhibit tumor progression (18), and the variability of concurrent inflammatory conditions.

Further work is also necessary to determine the exact pathways by which Neu5Gc is taken up into tissues, and potential approaches to prevent or eliminate such incorporation are being explored. The current findings indicate that this unique example of xenosialitis driven by a dietary glycan is pathologically relevant and may be involved in inflammation-driven diseases that are known to be associated with red meat consumption, including carcinomas.

Materials and Methods

Food Collection and DMB-HPLC Analysis. Sixty-two different types of food products (raw meat and three types of cooked meats, including baked, boiled, and fried foods, were sampled) were purchased from multiple local supermarkets, lyophilized, and pulverized. Samples were treated with 0.1 M NaOH at 37 °C for 30 min for the removal of O-acetyl groups and then neutralized. Acid hydrolysis of sialic acids from the underlying glycoconjugate was performed with 2 M acetic acid at 80 °C for 3 h. The samples were cooled to room temperature and centrifuged at 14,000 × *g* for 10 min, and the supernatant was filtered through a Millipore Ultra Centrifugal Filter. Aliquots of nonhydrolyzed samples were adjusted to 2 M acetic acid but not heated. The samples were then incubated with the two different derivatizing conditions (below) and analyzed by HPLC as described below.

DMB Derivatization Conditions. The DMB reagent, as per the conventional method (31, 46), was made with the following recipe and hydrolyzing conditions: 14 mM DMB, 18 mM sodium hydrosulfite, 0.75 M 2-mercaptoethanol, and 1.6 M acetic acid, and it was incubated at 50 °C for 2.5 h. The DMB reagent used for the quantification of free sialic acids was prepared as follows (32): 14 mM DMB, 18 mM sodium hydrosulfite, 1 M 2-mercaptoethanol, and 40 mM trifluoroacetic acid, and it was incubated at 4 °C for 48 h.

HPLC Analysis. The DMB-derivatized samples were analyzed on a Dionex Ultra3000 HPLC System using a Phenomenex Gemini 5μ C18 250 × 4.6-mm HPLC column at room temperature. The fluorescence was detected at 448 nm using excitation at 373 nm. For the separation of sialic acids that might coelute, for example, 3-deoxy-D-manno-octulosonic acid and 3-deoxy-D-manno-octulosonic acid, an isocratic solvent composition of 88% water, 7% methanol, and 5% acetonitrile was used. The data collection time was expanded to 90 min. Standard samples were run each day to account for slight variations in elution time.

Mice and Chow. *Cmah*$^{-/-}$ mice were bred in a congenic C57BL/6 background and maintained in the University of California, San Diego vivarium according to Institutional Review Board guidelines for the care and use of laboratory animals. *Cmah*$^{-/-}$ mice were maintained on a Neu5Gc-deficient soy-based chow (110951 for adults and 110751 for pregnancy/weaning; Dyets, Inc.), a Neu5Gc-rich soy-based chow (custom order containing 0.25 mg of Neu5Gc per gram of chow; Dyets, Inc.) made by adding purified PSM, or a Neu5Ac-rich soy-based chow (custom order containing 0.25 mg of Neu5Ac per gram of chow; Dyets, Inc.) made by adding EBN. For the preparation of PSM, cryoground porcine submaxillary glands (Pel-Freez Biologicals) were homogenized overnight in 5 vol of water and centrifuged at 8,000 × *g* for 15 min, and the supernatant was filtered through glass wool. The mucin was precipitated by gradual acidification (to pH 3.5) at 4 °C and left to settle overnight. The supernatant was removed by siphoning, and the precipitated mucin was centrifuged at 400 × *g* for 15 min, washed with water, and centrifuged again. Mucin pellets were neutralized to pH 8.0 and dialyzed using a 10,000 molecular weight cutoff membrane (Spectrum Labs) against 20 vol of water, with at least five volume changes. This final preparation was lyophilized, and its Neu5Gc content was quantified by DMB-HPLC. EBN was purchased from Golden Nest, Inc. and lyophilized to remove excess moisture. The dry EBN was pulverized into a fine powder, and acid hydrolysis for DMB derivatization was performed as mentioned above. It must be noted that the addition of PSM or EBN does not significantly increase the caloric content of the chow.

Preparation of RBC Membranes and Polyclonal Anti-Neu5Gc–Rich "Immune" and "Control" Sera. Chimpanzee and human RBC lysis was performed using 30 vol of ice-cold lysis buffer containing 10 mM Tris·HCl and 1 mM EDTA (pH 7.4) followed by centrifugation at 15,000 × *g* for 15 min at 4 °C. Pelleted membranes were washed in the same buffer until colorless. Protein quantification was performed, and *Cmah*$^{-/-}$ mice were immunized with 100 μL of 2 mg/mL RBC membrane ghosts (mixed with an equal volume of Freund's complete adjuvant) per mouse by i.p. injection. Two booster doses using Freund's incomplete adjuvant with the same amount of immunogen were given 1 wk apart. Two weeks after the second booster dose, serum was collected for ELISA analysis of anti-Neu5Gc response as described below. Positive sera were pooled, and nonspecific anti-RBC reactivity was removed by repeated adsorption against human RBCs. Adsorption was performed using 100 μL of packed washed human RBCs incubated with pooled immune or control sera at 4 °C for 2 h, and the RBCs were subsequently removed by centrifugation. The adsorption was repeated three times. The pooled serum from the human RBC-immunized mice was processed in exactly the same way. The mouse anti-Neu5Gc IgG was quantitated using the ELISA method described below. Based on the above method, these antibodies should comprise the primary difference between the immune and control sera.

ELISAs for Detection of Anti-Neu5Gc Antibodies in Mice. Microtiter plate (Costar 9018) wells were coated with Neu5Gc-rich bovine submaxillary mucin in the amount of 1 μg per well in 50 mM sodium carbonate-bicarbonate buffer (pH 9.5) at 4 °C overnight. After washing with PBS (pH 7.4) and blocking with PBS containing 1% Neu5Gc-free chicken ovalbumin for 1 h at room temperature, triplicate wells were incubated with 1:100 dilutions of mouse serum in PBS containing 1% chicken ovalbumin at room temperature for 2 h. Wells were washed five times with PBS containing 0.05% Tween 20 (Fisher-Scientific) and incubated with HRP-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories), diluted in PBS at 1:7,000, at room temperature for 1 h. Anti-Neu5Gc IgG was quantified using a standard curve of normal mouse IgG coated to the wells under the same general conditions. To confirm the specificity of anti-Neu5Gc antibodies, mild periodate treatment was used selectively to cleave the Sia side-chain and eliminate specific reactivity, as described previously (47).

Measurement of Peritoneal Lavage Fluid IL-6, Serum Amyloid A Protein, and Haptoglobin. Commercially available kits were used for the measurement of inflammatory markers, peritoneal lavage fluid IL-6 (no. DY406, Mouse Il-6 DuoSet; R&D Systems), serum haptoglobin (no. 2410-1; Life Diagnostics, Inc.), and serum amyloid protein A (no. TP 802M; Tridelta) according to the manufacturers' instructions.

Neu5Gc Detection by Immunohistochemistry. Tissues from *Cmah*$^{-/-}$ mice fed or not fed PSM were flash-frozen in optimum cutting temperature compound (Sakura), and frozen sections were air-dried overnight at room temperature and then immersed in PBS containing 0.1% Tween (PBST) wash buffer. The sections on the slides were treated to eliminate endogenous biotin and endogenous peroxidase, and postfixed in 10% neutral buffered formalin (Fisher), washing between each step. Positive controls on separate slides included sections from WT mouse liver, and negative controls included sections from *Cmah*$^{-/-}$ mouse livers. The negative controls used competitive inhibition of anti-Neu5Gc binding, by incubating 20% chimpanzee serum with the anti-Neu5Gc antibody for 1 h at 4 °C before overlaying on serial frozen sections. All slides were incubated in a humid chamber for at least 1 h at room temperature

with anti-Neu5Gc IgY or with the control IgY (Sialix, Inc.) at 5 μg/mL [1:2,000 diluted in blocking buffer, 0.5% cold water fish skin gelatin (Sigma) in PBST]. Slides were then overlaid successively with the biotinylated donkey anti-chicken IgY (1:500; Jackson ImmunoResearch) and with HRP-streptavidin (1:500; Jackson ImmunoResearch) for 30 min each. Substrate color development used 3-amino-9-ethylcarbazole (Vector Laboratories AEC substrate kit), and the nuclei were counterstained with Mayer's hematoxylin (Sigma). Slides were coverslipped using Aqueous VectaMount (Vector Laboratories) for viewing, and digital photomicrography was performed using a Keyence B6000 microscope.

Statistical Analyses. Statistical analyses were performed on GraphPad Prism version 6.0 or Microsoft Office Excel. Relevant tests of significance and the statistical tests used are further detailed in the figure legends.

ACKNOWLEDGMENTS. We thank Profs. Walter Willett (Harvard University) and Kay-Tee Khaw (Cambridge University) for helpful discussions, and Michelle Y. Chung, Pratyash Srivastava, and Siavash Assar for help with some of the experiments. This work was supported by a Samuel and Ruth Engelberg Fellowship from the Cancer Research Institute (to O.M.T.P.), the Swiss National Science Foundation (H.L.), the Ellison Medical Foundation (A.V.), and NIH Grant R01CA38701 (to A.V.).

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