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A GLYCOFORM OF IMMUNOGLOBULIN G (IgG) AS AN EARLY BIOMARKER OF EXPOSURE TO NONHUMAN SUBSTANCES

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PREFACE

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A GLYCOFORM OF IMMUNOGLOBULIN G (IgG) AS AN EARLY BIOMARKER OF EXPOSURE TO NONHUMAN SUBSTANCES

1. INTRODUCTION

1.1 Overview

The ultimate goal of this research was to develop a blood serum assay similar to an enzyme-linked immunosorbent assay (ELISA) to rapidly determine whether an individual or animal has had a recent exposure to an agent of bioterrorism or other health concern. Such methods are critical for alerting a caregiver that an individual has been exposed to a foreign substance, and the sooner a suspected exposure is validated, the better the medical outcome. We have shown that immunoglobulin G (IgG), produced ≤ 7 days after animals are exposed to a range of antigens, contains altered *N*-linked glycans, and further, we have observed this with human test patients as well. The alteration renders the IgG in its native conformation to be readily detectable by certain lectins, a condition under which most IgGs are not detectable by lectins. Because the altered IgG is produced within days following exposure, apparently regardless of the antigen, and is easily detected in serum by ELISA-type methods, it can serve as the basis of an early detection method for exposure of humans to antigens, even when the identity of the antigen is not known. Herein, we describe the extent to which our observation is general and whether such an assay can be developed. Generally speaking, this should have great value for the Government's biodefense effort, for use in community health settings and in basic research and science.

1.2 Technical and Scientific Details

We have identified an IgG subset that transiently exists in the blood of rabbits and mice following the introduction of various nonself antigens. This previously unrecognized IgG has been named "primebody" because it appears to be involved in priming the humoral immune response rather than in neutralizing immunogens. Primebody was initially identified by its ability to bind the *Aleuria aurantia* lectin (AAL) while it is still in its native conformation. In contrast, most serum IgGs bind AAL poorly, unless the IgG is first denatured. For this effort, we developed an immunogen-independent, lectin-antibody microarray assay that uses F(ab')₂ anti-IgG as a capture reagent and a biotinylated AAL as a detection reagent to measure the serum primebody level. We detected primebody in all serum samples from animals inoculated with various immunogens, including ovalbumin (OVA), kininogen (KIN), and inactivated rabies virus (RV), both with and without adjuvants. These animals subsequently developed antigen-specific, humoral immune responses. The production kinetics of primebody in mice and rabbits were distinct from those of the immunogen-specific IgG produced in the same animals. Specifically, primebody levels rose and fell within 1 to 2 weeks of exposure and peaked between 4 and 7 days after exposure, and IgGs specific for the stimulating immunogen continued to rise during this same period. Moreover, higher levels of primebody during the early immune response correlated well with a higher level of the

immunogen-specific IgG observed late in the immune response, suggesting that primebody could be involved in the priming-specific IgGs as part of the primary response. Mass spectrometry (MS) profiling of the glycoforms, derived from the AAL-purified IgG and total serum IgG from immunized rabbits, indicated that primebody is mainly comprised of constant region fragment (Fc) glycoforms that are core-fucosylated and either mono- or non-galactosylated *N*-glycans. Primebody could be an IgG subset that plays an important role in immune responses. Additionally, it may be a potentially universal biomarker that alerts the immune system upon exposure to a foreign entity.

2. EXPERIMENTAL INVESTIGATION AND RESULTS

2.1 Primebody Detection and Quantification Using AAL-Antibody Microarrays

We previously reported that most native serum IgGs do not bind AAL. Specifically, AAL binding by IgG follows its denaturation, presumably because the fucose on the IgG is normally inaccessible to the lectin (1). Heating, or the use of denaturation reagents such as urea, can fully denature the IgG protein and expose the fucose moieties. In addition, glycosidase treatment or solid-surface immobilization can partially denature the protein and expose the IgG glycans. The apparent disassociation constant (K_d) for AAL binding to immobilized IgG was estimated at 150 nM (2), but this was not measurable for IgGs in solution phase. There was, however, a subset (<0.1%) of the total, native IgG that could bind AAL, even prior to denaturation.

To measure the subset of AAL-reactive IgGs in different samples, we developed a lectin-antibody, sandwich-type, affinity-based assay in which anti-IgG antibodies were, or protein A/G was immobilized on a solid surface to capture serum IgGs, and biotinylated AAL was used to detect the glycans on the captured IgGs. We first used a microplate meant for conventional ELISAs, but found that the sensitivity of the AAL-antibody assay was too low for detecting primebody in normal serum samples. When we tested the AAL-antibody detection assay with a high-density antibody microarray format, the detection sensitivity was significantly increased and adequate for primebody detection. This may have been due to the contribution of an increased number of IgG capture epitopes on the antibody microarrays (ultrathin nitrocellulose) compared with the normal ELISA microplates (polystyrene or polycarbonate). The antibody microarray also allowed a very high density of captured antibodies (3,4).

We tested multiple capture reagents, including goat and donkey F(ab')₂ as well as recombinant protein A/G, to capture mouse or rabbit IgG and identified donkey F(ab')₂ antimouse and antirabbit IgGs as giving the highest signal-to-noise ratios in the standard curves shown in Figures 1B and 1C. There is no standard AAL-reactive IgG that can be used as a reference for the calculation of an absolute concentration for primebody. Therefore, to normalize our results and obtain semi-quantitative results for primebody, we used completely denatured IgG with fully accessible fucose residues as a reference to measure the fucose levels in the serum IgG. This standard, together with a known IgG concentration, allowed the estimation fucose levels of the serum IgG by converting AAL

binding (fluorescence intensities of Dylight 549 Neutravidin, Pierce Biotechnology, Rockford, IL) to the concentration of primebody as illustrated in Figure 1A. Standard curves were prepared using completely denatured mouse (Figure 1B) and rabbit (Figure 1C) IgG for the quantitation of mouse or rabbit primebody, respectively. The limit of detection for both assays was ~ 10 ng/mL, which was lower than that of conventional sandwich ELISA assays, but acceptable for normal primebody detection. Considering that primebody still maintains its native structure and the AAL-binding affinity may be different from that of completely denatured IgGs, we used these standard curves to semi-quantify the native primebodies. Because the lowest dilution factor that did not saturate the assay was 1:100, the actual AAL-reactive IgG detection limit was $1 \mu\text{g/mL}$ using this assay. Thus, we defined $1 \mu\text{g/mL}$ as the minimal detection limit for primebody. Taken together, the AAL-binding assays using microarrayed immobilized IgGs were quantitative.

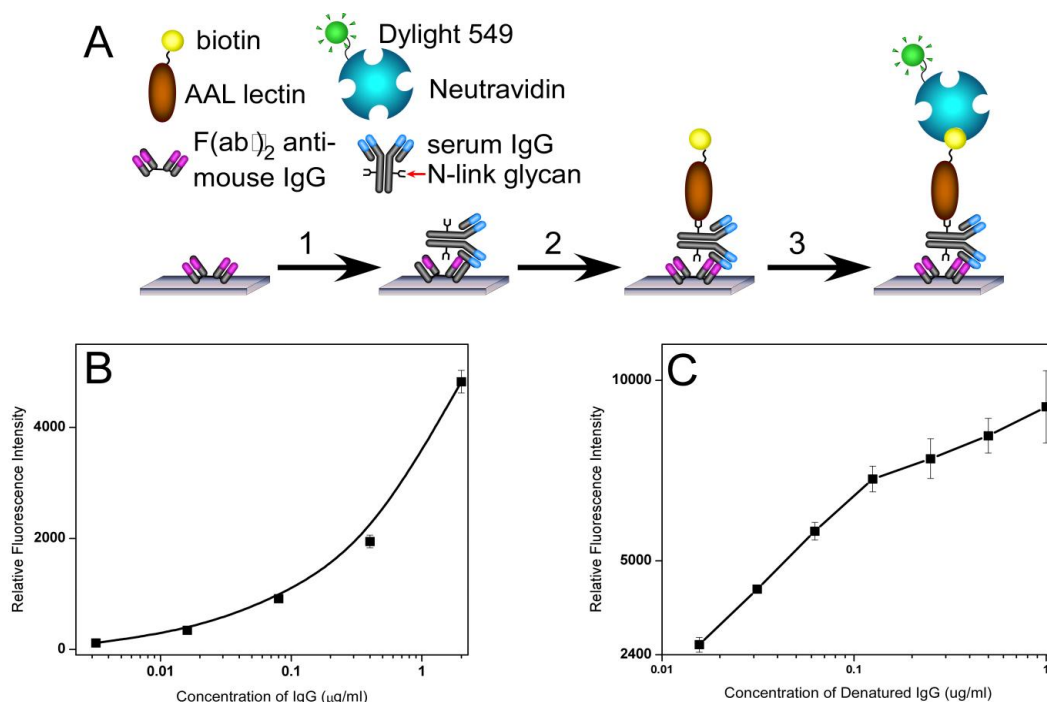


Figure 1. Measurement of primebody using AAL-antibody microarrays. (A) Schematic of the AAL-antibody microarray protocol used for primebody detection: (1) mouse or rabbit serum samples were incubated on an antibody microarray, (2) AAL was applied to the microarray to detect the glycans on captured IgGs after unbound protein was removed by washing, and (3) Dylight 549 NeutrAvidin was incubated on the microarray to detect biotinylated AAL. (B) Representative mouse primebody standard curve for calculating primebody concentration. Denatured mouse IgG of known concentration was used as a standard, with goat $F(ab')_2$ antimouse IgG as a capture reagent. (C) Representative rabbit AAL-reactive IgG standard curve for AAL-reactive IgG concentration calculation. The standard was denatured rabbit IgG at known concentration, and the capture reagent was a donkey $F(ab')_2$ antimouse IgG.

2.2 Primebody and Immunogen-Specific IgG Produced in the Same Animal Exhibit Different Kinetics

As mentioned in Section 2.1, >90% of the Fc *N*-glycans in mouse and human IgGs are core-fucosylated but cannot bind AAL when present on intact IgG. The ability to bind AAL usually requires either denaturation or treatment with glycosidases to expose the core fucose residues to the AAL. However, Mehta et al. (1) discovered specific IgGs that can be bound by AAL, even when in its native conformation. Furthermore, the IgGs were greatly elevated in the serum of human patients with liver diseases such as cirrhosis. This led to speculation that the IgGs were produced in response to antigens from microbial translocations of the gastrointestinal track because their predominant epitope was 1,3- α -galactose (1). Therefore, we proposed that the AAL-reactive IgGs, or primebody, could be a general property of IgG that was generated during the early immune response to any antigen.

We first tested this hypothesis by inoculating five BALB/c mice with 50 μ g of OVA in phosphate-buffered saline (PBS) and monitoring the primebody production kinetics using the method described in Section 2.1. Due to the expected lack of adjuvant, however, neither OVA-specific IgG nor primebody was detected in the mice. This result suggests that primebody was not produced without establishment of an adaptive immune response.

We then immunized five different BALB/c mice with 100 μ g OVA in incomplete Freund's adjuvant (OVA/IFA), collected blood samples from the mice by orbital bleeding, and prepared serum samples at serial time points prior to days 7 and 0 and post immunization (day 1, 3, 5, 7, 10, 12, 15, 21, 30, 40, 50, 60, 70, 80, 90, 100, 120). These mice are listed in Table 1 along with the other animals, immunogens, and routes of entry used in this study. The kinetics of OVA-specific IgG and primebody in the five inoculated mice and one control mouse were monitored using the antibody microarray described in Section 2.1. As shown in Figure 2B, the control mouse did not produce AAL-reactive or OVA-specific IgGs at any of the times tested during the entire period of immunization. However, both primebody and OVA-specific IgG were detected in response to the OVA inoculation as shown in Figure 2A. Interestingly, the kinetics of the primebody and OVA-specific IgG were characteristically different. Analysis of the OVA-specific IgG produced a typical IgG development curve, the level of which rose consistently from day 0, peaked at day 60, and then fell to essentially baseline levels at day 100. Contrary to the OVA-specific IgG results, the primebody levels quickly rose within 3 days, peaked at day 5 following inoculation with OVA/IFA, and fell rapidly at a rate similar to its earlier rise. These results indicate that primebody did transiently appear in serum with unique wave kinetics in the early stage of the immune response and exhibited a kinetics curve distinct from the immunogen-specific IgG produced in the same animal.

Table 1: Animals, Immunogens, and Routes of Inoculation in this Study

Animal	Group	Species	Gender	Number of Animals	Immunogen	Dosage per Animal	Routes
mouse	1	BALB/c	female	5	OVA/IFA ^a	100 µg	IP ^b
mouse	2	BALB/c	female	5	KLH ^c	100 µg	IP
mouse	3	BALB/c	female	1	N/A ^d	N/A	N/A
mouse	1	C57/BL6	female	5	OVA/CFA ^e	100 µg	IP
mouse	2	C57/BL6	female	5	RV ^f (108 ffu)	100 µL	IP
rabbit	1	NZW ^g	male	2	OVA/CFA	500 µg	subQ ^h
rabbit	2	NZW	male	2	KIN ⁱ /CFA	500µg	subQ
rabbit	3	NZW	male	1	N/A	N/A	N/A

- ^a ovalbumen/incomplete Freund's adjuvant
^b IP, intraperitoneal injection
^c KLH, keyhole limpet hemocyanin
^d N/A, not applicable
^e CFA, complete Freund's adjuvant
^f RV, rabies virus inactivated by ultraviolet light exposure
^g NZW, New Zealand White
^h subQ, subcutaneous injection
ⁱ KIN, kininogen

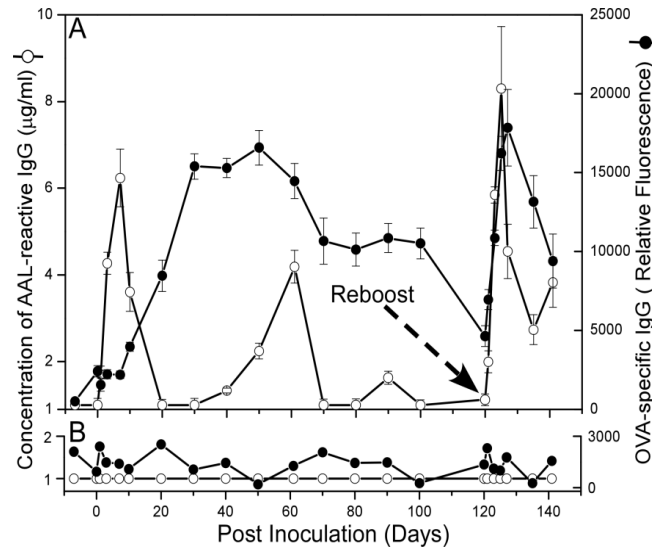


Figure 2. Production kinetics of primebody and OVA-specific IgG in OVA/IFA-immunized mice determined with AAL-antibody microarrays. Inoculated mice were reboosted at day 120. (A) AAL-reactive IgG and OVA-specific IgG production kinetics of OVA/IFA inoculated mice. Each point represents the average AAL-reactive concentration from five inoculated mice, calculated according to AAL-reactive IgG standard curve. (B) AAL-reactive IgG and OVA-specific IgG production kinetics of a control mouse.

2.3 Primebody is Detected in Animals Inoculated with OVA or KIN Adjuvants or with Killed Rabies Virus Alone

To determine whether primebody generation occurs regardless of the antigens or animal species and strains, we carried out animal immunization experiments using different mouse strains and rabbits, inoculating them with different antigens as summarized in Table 1. C57BL/6 mice were inoculated with either OVA/CFA or with RV. Two rabbits were inoculated with OVA/CFA, and another two were inoculated with KIN/CFA. Blood samples from these animals were analyzed to determine the levels of primebody and immunogen-specific IgG as described in Section 5.

As shown in Figures 3A (C57BL/6 mice inoculated with RV), 3B (C57BL/6 mice inoculated with OVA/CFA), 3C (rabbits inoculated with OVA/CFA, and 3D (rabbits inoculated with KIN/CFA), the rapid rise-and-fall kinetics characteristic of primebody was observed in all animals regardless of the antigens used in the inoculations or the species or strains of the animals. Primebody was not detected in the control animals that were not inoculated. The exact time of the peak primebody levels varied slightly, but generally appeared between days 6 and 10. Specific IgGs were produced as would be expected of a typical response curve for all animals, suggesting that immune responses were elicited. Therefore, the occurrence of primebody can be a common phenomenon in animals as a result of an immune response, regardless of the types of animals or immunogens.

The exact day after immunogen inoculation that the primebody levels peaked varied with the species and immunogen. For example, the primebody level peaked at day 4 in mice inoculated with RV, at day 12 in the mice inoculated with OVA, and at day 9 in the rabbits inoculated with OVA or KIN. This peak difference can be correlated with both the animal species and the immunogens because there was no individual variation within the groups.

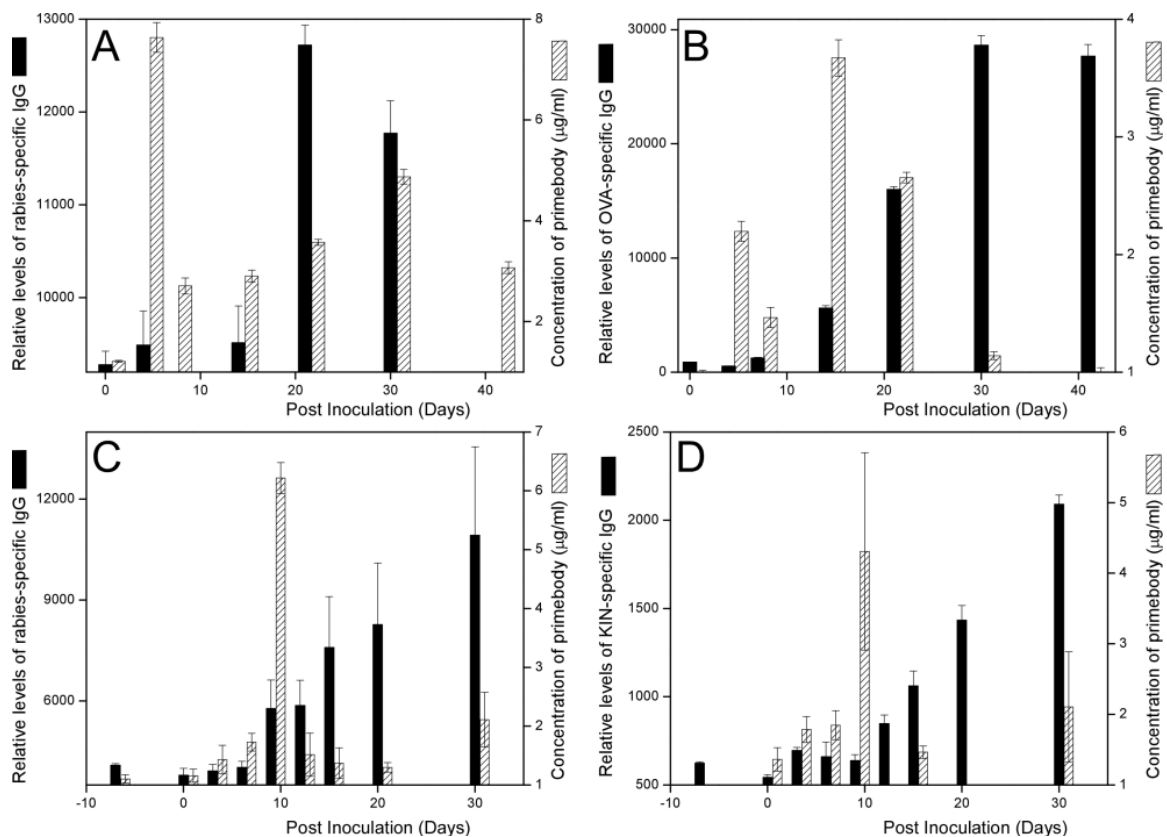


Figure 3. Production kinetics of AAL-reactive and immunogen-specific IgG in mice and rabbits. Kinetics were determined with AAL-antibody microarrays. Different animals were immunized with different immunogens as shown in Table 1. Immunogen-specific IgG and primebody were measured in serum samples taken from each animal. Black bars are averaged, immunogen-specific IgG levels (relative levels according to fluorescence intensities), and striped bars are averaged primebody levels: (A) five C57BL/6 mice inoculated with inactivated RV, (B) five C57BL/6 mice inoculated with OVA/CFA, (C) two rabbits inoculated with OVA/CFA, and (D) two rabbits inoculated with KIN/CFA.

2.4 Peak Primebody Levels Correlate with Peak Antigen-Specific IgG Levels

We found that peak primebody levels correlated well with antigen-specific IgG levels in the same animal. In immunization experiments, two groups of two rabbits each were inoculated with either OVA/CFA (Figures 4A and 4C) or KIN/CFA (Figures 4B and 4D). The primebody and the KIN- or OVA-specific IgG levels were measured using the lectin-antibody microarrays as described in Section 5. Figure 4A shows that the primebody levels peaked similarly in both of the two OVA/CFA-inoculated rabbits. Moreover, the peak levels of the immunogen-specific IgG occurred at day 30 for both rabbits (Figure 4C), well after the primebody levels peaked. Similar kinetics for primebody and immunogen-specific IgGs were also observed in the KIN/CFA-inoculated rabbits. As revealed in Figure 4B, rabbits 4 and 5 were both inoculated with KIN/CFA and their primebody levels peaked at day 9, with the response of rabbit 4 more than twice that

of rabbit 5. Additionally, the KIN-specific level of rabbit 4 was more than twice as high as that of rabbit 5 (Figure 4D). These results confirmed that the correlation between primebody and immunogen-specific IgG was not a random event. Inducing more primebody at the beginning of the immune response leads to stronger immunogen-specific, adaptive immune response.

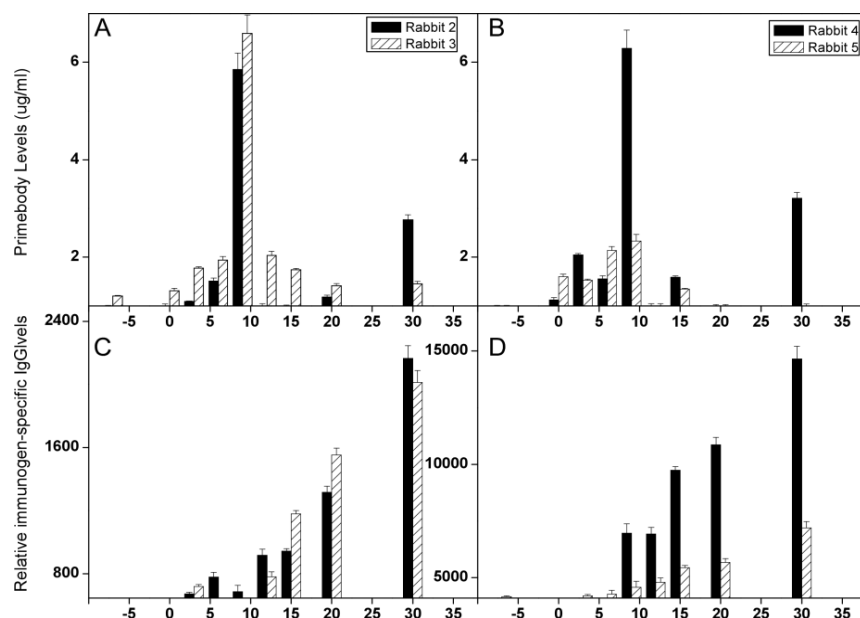


Figure 4. Correlation of peak levels for primebody and immunogen-specific IgG. Rabbits 2 and 3 were inoculated with OVA/CFA while rabbits 4 and 5 were inoculated with KIN/CFA. The kinetics of OVA- or KIN-specific IgG and primebody in each rabbit were monitored and plotted. The bar graphs show (A) primebody kinetics in OVA/CFA-inoculated rabbits, (B) primebody kinetics in KIN/CFA-inoculated rabbits, (C) OVA-specific IgG kinetics in OVA/CFA-inoculated rabbits, and (D) KIN-specific IgG kinetics in KIN/CFA-inoculated rabbits.

2.5 Primebody Has Low Affinity for Its Immunogen

To understand the biological function of primebody, we investigated its binding affinity for its immunogens. Standard curves were generated from data that were derived from incubating serially diluted, purified serum primebody with immobilized immunogens. We defined the 50% effective concentration (EC_{50}) as the apparent K_d and used this value to quantify binding affinities.

Primebody (in AAL immunoprecipitation pull down) and OVA-specific IgG (OVA immunoprecipitation pull down) were purified separately and their affinities for OVA were measured using a sandwich microarray. $F(ab')_2$ antirabbit IgG and OVA were immobilized on the microarrays for determining the concentration of total IgG and OVA-bound IgG in a serial dilution of the primebody and OVA-specific IgG. The binding

curves of primebody and OVA-specific IgG were then plotted as the relative fluorescence of their signals as a function of their concentrations. The apparent K_d values of the curves were estimated to represent EC_{50} values. Figure 5 shows that although the plateaus of the curves were not reached in the experiments, the apparent K_d value of primebody was estimated to be ~100-fold lower than that of the OVA-specific antibody. Therefore, although primebody and OVA-specific IgG were produced from the same rabbit in response to the OVA inoculation, the immunogen binding affinity of the primebody was significantly lower than that of the OVA-specific IgG.

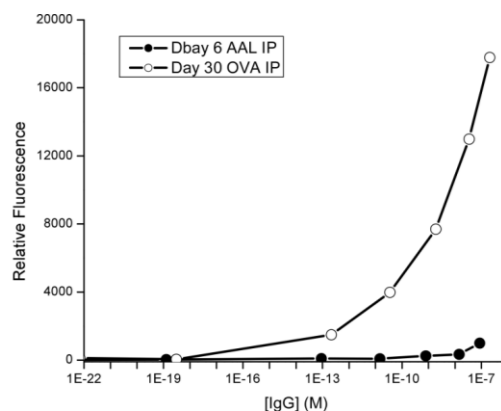


Figure 5. Comparison of OVA binding affinity for primebody and OVA-specific IgG at peak levels. Primebody and OVA-specific IgG were purified from an OVA/CFA inoculated rabbit (rabbit 4) using OVA-agarose and AAL-agarose bead immunoprecipitation as described in Section 5. Primebody was purified from the day 9 serum sample while OVA-specific IgG was purified from the day 30 serum sample. These were serially diluted and applied onto a protein-antibody microarray containing immobilized OVA and donkey F(ab')₂ antirabbit IgG spots. The bound IgG levels on OVA and the IgG concentration were measured simultaneously. The OVA-bound primebody and OVA-specific IgG, measured with immobilized donkey F(ab')₂ antirabbit IgG, were plotted as a function of their concentrations.

2.6 Primebody Contains Core-Fucosylated, Under-Galactosylated Fc *N*-Glycan

Because the AAL specifically binds fucosylated proteins or oligosaccharides (5), it was likely that the primebody itself is fucosylated. Therefore, we profiled primebody IgG Fc glycoforms of purified primebody using MS techniques to evaluate whether this is true.

MS Fc glycoform profiling has been performed by many groups by taking advantage of the fact that only one *N*-linked glycosylation site exists at Asn 297 of the immunoglobulin constant region heavy domain 2 (C_H2). Thus, the identity and content of each Fc glycoform in IgG can be determined through MS analysis because two moles of glycopeptide are equivalent to one mole of IgG. Because the total amount of mouse serum collected was insufficient for glycoform-profiling assays, we purified primebody from

rabbit 4, which was inoculated with OVA/CFA (Table 1) using an AAL-agarose protocol followed by Melon gel purification as described in Section 5. Serum incubated with “empty” agarose beads was used as a control. The purified primebody and control samples were denatured, trypsin digested, and finally injected into a nanoflow liquid chromatography tandem MS (LC-MS/MS) instrument (ABI QStar Elite, Applied Biosystems, Life Technologies Corporation, Carlsbad, CA) for glycoform identification and quantification as described in Section 5 and Figure 6A. Due to the hydrophilic nature of the glycan moieties, glycopeptides were always eluted from the reverse-phase column at the beginning of the LC gradient (14 to 15 min as shown in Figure 6B). For quantification and kinetic profiles of each IgG Fc-glycoform, MS-only analysis allowed only the precursor ions to be analyzed (Figure 6C). The extracted ion chromatogram (XIC) for each glycosylation subtype was plotted, and the relative abundance for each was represented by the peak area in each XIC (Figure 6D and 6E).

To determine the glycan structure for each glycopeptide, tandem MS (MS/MS) analysis was performed on each peak to identify all glycoforms. Figure 6E is an example MS/MS spectrum of G0 glycopeptide. Twenty-two different IgG glycoisoforms, identified in the rabbit IgG samples are listed in Table 2, and all are from the same peptide sequence. Quantification of each IgG glycoisoform was calculated by using the integrated peak area of all ions (+2, +3) from the corresponding glycopeptides in MS spectra. Nine of the 22 glycoforms were fucosylated while the rest were not. In the nonimmunized rabbit (day 0), the Fc-fucosylated IgGs were ~18.8%, which was consistent with that reported previously. However, the major IgG glycoforms in primebody were core-fucosylated IgGs (84.5%) and nonfucosylated IgGs (15.5%). This was significantly higher than that in the nonimmunized rabbit (~9% on average). In core-fucosylated IgGs, the majority were fucosylated G0, G1, and their bisecting forms; fucosylated G2 or bisect G2 were not found. The main glycoisoforms in primebody are fucosylated IgGs, such as G0F and G0BF, although they were not the dominate glycoforms found in rabbit serum IgGs. Taken together, these results suggests that primebodies are mainly a mixture of IgG glycoisoforms that possess core-fucosylated and low-galactosylated *N*-linked structures.

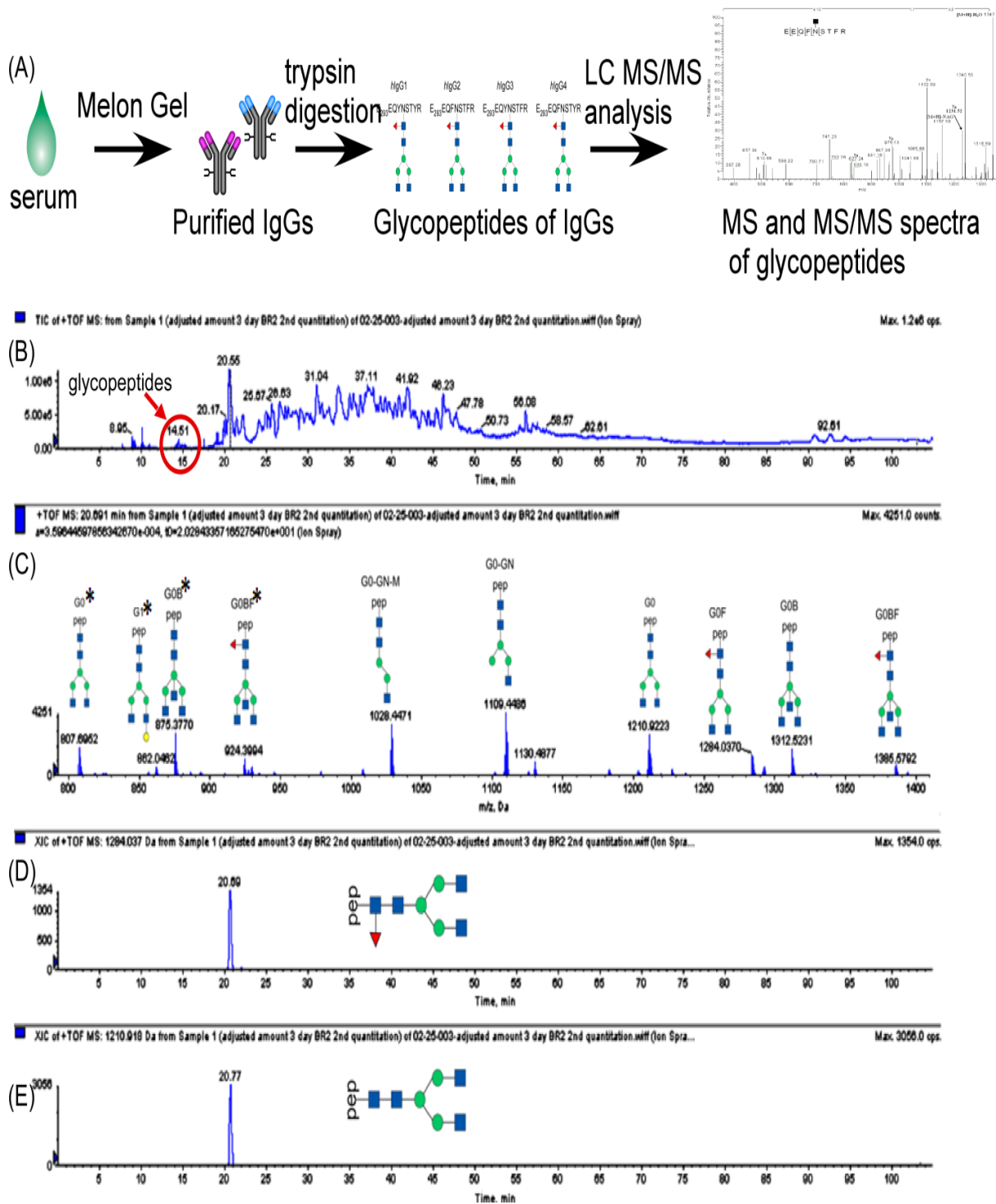


Figure 6. MS identification and glycoform profiling of IgG and primebody. (A) scheme for the procedure (see details in Section 5), (B) total ion chromatograph of purified rabbit IgG, (C) mass spectrum of the sample at retention time 19 min, and (D) and (E) XICs of G0F and G0, respectively (Figure continued on next page.)

(F)

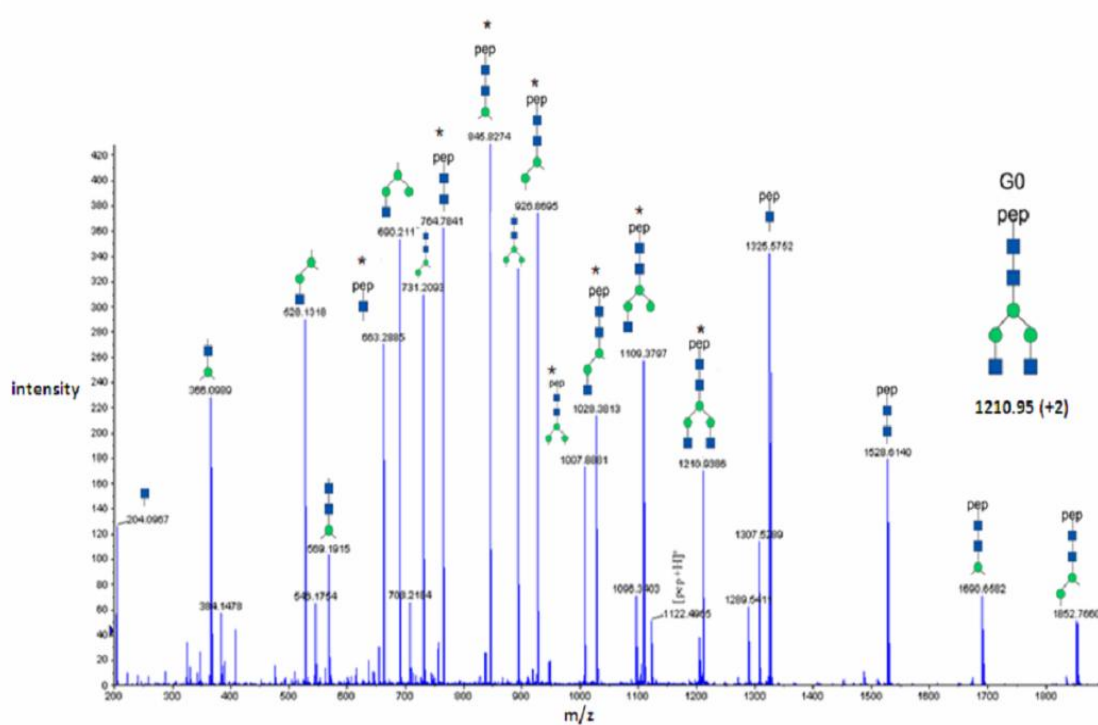


Figure 6. MS identification and glycoform profiling of IgG and primebody (continued). (A) scheme for the procedure (see details in Section 5), (B) total ion chromatograph of purified rabbit IgG; (C) mass spectrum of the sample at retention time 19 min; (D) and (E) XICs of G0F and G0, respectively; and (F) MS/MS spectrum of G0 IgG.

Table 2. MS Characterization of Fc Glycoforms for Total IgG and Primebody Purified from OVA-Inoculated Rabbits

Number	Name	Glycan Composition	2 ⁺ m/z	3 ⁺ m/z	Content (%)	
					Non-Immunized Rabbit	Purified IgG ^a
1	G1F	H4N4F1	1365.05	910.37	3.4	22.7
2	G1BF	H4N5F1	1466.50	978.00	1.8	13.8
3	G0F	H3N4F1	1284.03	856.35	6.2	12.2
4	G0F-GlcNAc	H3N3F1	1182.50	788.67	1.9	11.8
5	G0BF	H3N5F1	1385.56	924.04	3.2	11.7
6	G2	H5N4	1373.05	915.70	1.0	7.3
7	G1F-GlcNAc	H4N3F1	1263.50	842.67	0.5	6.2
8	G1B	H4N5	1393.50	929.33	3.1	6.1
9	G0F-GlcNAc-Man	H2N3F1	1101.05	734.67	0.8	3.7
10	G1FNeu5Gc	H4N4F1S1	1518.63	1012.75	0.4	3.2
11	G2Neu5Gc	H54S1	1474.55	983.45	0.5	0.7
12	G2FNeu5GC	H5N4F1S1	1547.67	1032.11	0.3	0.7
13	G0-2GlcNAc-Man	H2N2	926.90	618.27	1.4	0.0
14	G0-2GlcNAc	H3N2	1007.90	672.27	1.3	0.0
15	G0-GlcNAc-Man	H2N3	1028.45	685.97	8.8	0.0
16	G0-GlcNAc	H3N3	1109.43	739.95	16.3	0.0
17	G1-GlcNAc	H4N3	1190.48	793.99	2.3	0.0
18	G0	H3N4	1210.95	807.63	20.0	0.0
19	G0B	H3N5	1312.52	875.35	16.2	0.0
20	G1	H4N4	1291.94	861.63	9.5	0.0
21	G2F	H5N4F1	1446.08	964.39	0.4	0.0
22	G1Neu5Gc	H4N4S1	1445.58	964.05	1.4	0.0
Total Fucosylated Glyco Isoforms					18.8	84.5
Total Nonfucosylated Glyco Isoforms					81.2	15.5

^a Purification by AAL-immunoprecipitation pulldown

2.7 Primebody Kinetics Correlates with the Kinetics of Core-Fucosylated, Under-Glycosylated IgG

To further address primebody structure and total IgG glycoform structural alterations during an immune response, we quantified the glycoform content of total IgG during a rabbit immune response to KIN/CFA. Briefly, we purified total rabbit serum IgG from rabbit 4 (Table 2) at pre-immunization day 7 and at post-immunization days 0, 3, 6, 9, 12, 21, 30, and 40, and determined the total IgG glycoforms at each time point by MS. Because the concentration of total IgG changed insignificantly, the percentile changes of the IgG glycoisoforms reflected the dynamics of the metabolism of certain isoforms (decrease) and the production of new isoforms. The kinetics of primebody, which was the

sum of the primebody glycoform content shown in Table 2, and the primebody kinetics measured using an AAL microarray assay were overlaid together for the comparison shown in Figure 7. The glycoform levels of primebody gradually rose from day 3, peaked at day 9, and rapidly fell to background level during days 9 to 12. The two curves produced from two different methods were very similar, which indicated that the primebody measured with the simple AAL microarray assay reflected the IgG glycoform changes. This result confirmed that the results in the AAL immunoprecipitation for the fucosylated forms of the G0, G1, bisecting G0, and bisecting G1 glycoisoforms were the AAL-reactive.

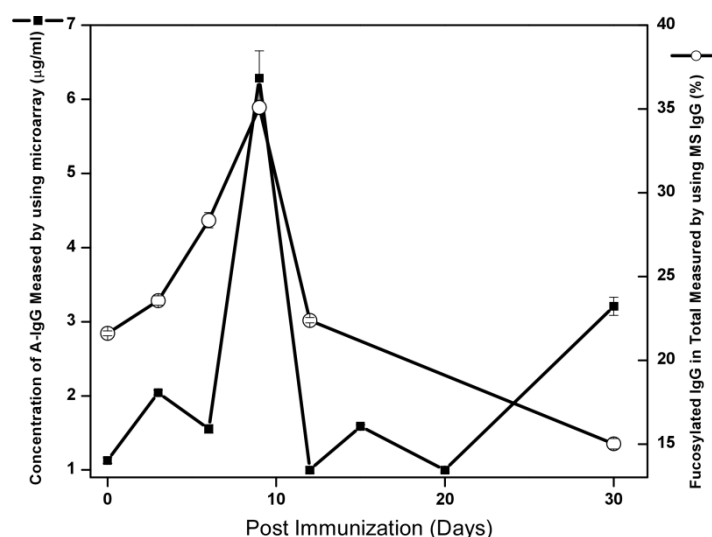


Figure 7. Kinetics comparison between rabbit AAL-reactive IgG and fucosylated IgG glycoforms. Kinetics were determined from AAL-antibody microarrays, and the amount of fucosylated IgG glycoforms were determined by MS. Data are from rabbit 4 inoculated with KIN/CFA (see Table 1). IgG glycoforms in the total IgG of the serum samples at each time point were profiled using the MS method described in Section 5.

3. DISCUSSION

To our knowledge, this is the first report of an undenatured IgG that reacts with AAL whose abundance in the circulation rises following antigen exposure. We refer to this IgG subset as *primebody* and describe the primebody production kinetics found in different animals inoculated with different antigens, as well as some of the primebody glycan structure and biological properties. However, the precise structure or biological properties of primebody remain undetermined, largely because primebody is a mixture of IgGs that possess different structures and antigen binding affinities. Therefore, the property that we use to define primebody is its AAL reactivity. From our MS results, we determined that this AAL-reactive IgG is a subset of undergalactosylated IgG (G0 and G1), with core fucose on its Fc *N*-glycan and with an open or flip-out immunoglobulin protein conformation that allows AAL access (Figure 8). In contrast, most immunoglobulins

assume a closed conformation that denies AAL access to the glycans. The closed conformation may explain why most serum IgGs typically do not bind AAL. It is unclear whether primebody is preferred to any IgG subclasses (IgG1, IgG2, IgG3, and IgG4) or to the helper T cell response.

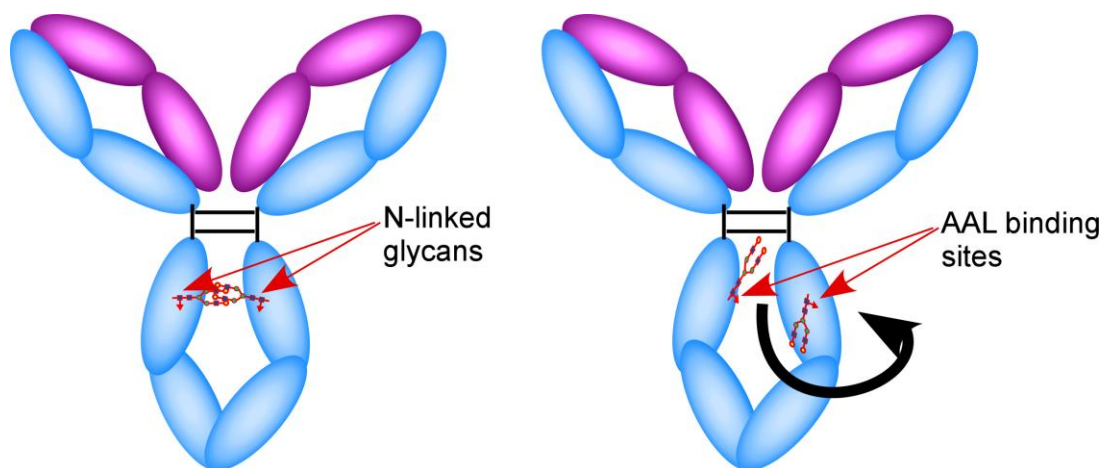


Figure 8. Hypothetical models of (left) non-AAL-reactive IgG in the closed conformation and (right) AAL-reactive IgG, or primebody, in the flip-out conformation.

Primebody was initially identified by its ability to bind AAL in its native conformation as most other serum IgGs bind AAL poorly unless they are denatured. AAL-primebody binding likely occurs through the glycan moieties of the IgGs because AAL specifically binds fucose residues. MS glycoform profiling of purified rabbit primebody confirmed that 84.5% of primebody is core-fucosylated. In comparison, core-fucosylated IgG in rabbit serum IgG is normally 18.8%. Fc glycoform profiling also indicated that primebody contained mainly undergalactosylated forms, in which 45.9% is the G1F, its bisecting form, and a form with a loss of one GlcNAc residue, while 39.4% is G0F, its bisecting form, and a form with a loss of one GlcNAc residue. These results confirmed that primebody contains a subset of fucosylated G0F or G1F IgGs. The AAL binding of primebody appears to be related to the conformation of the IgG C_H2 domain. Normally, Fc glycan is located at the domain close to the hinge region and stabilizes its conformation through glycan–glycan interaction between the two Fc glycans of the two heavy chains. The conformation buries the fucose inside the protein, resulting in its inaccessibility to AAL (Figure 8, left). This is the reason that primebody cannot be detected in normal human and mouse serum, as 90% of human and mouse IgGs are fucosylated. However, this closed structure could open to a flip-out conformation and expose fucose moieties to AAL. In support of this, a recent study using hydrogen–deuterium exchange confirmed the existence of this open IgG conformation. Therefore, we hypothesize that primebody is a subset of G0F/G1F IgGs that possess the open conformation, enabling AAL access to fucose moieties on Fc N-glycans (Figure 8, right).

Primibody represents such a small fraction of total serum IgG that its presence is detected only when using conformation- and glyco-specific methods. The production of relatively low levels of primebody (16 µg/mL) appears to be a common event during the early stage of adaptive immune responses, regardless of animal type or immunogens, as primebody was observed in the serum of all the animals inoculated with different immunogens. Therefore, primebody could play an essential role in a humoral immune response. The very weak binding to the primebody immunogen and the correlation between primebody levels and immunogen-specific IgG levels all suggest that primebody may be involved in priming the humoral response instead of neutralizing foreign antigens. However, more investigation is needed to determine whether primebody is generated in a cell-mediated immune response.

The unique structure, biological function, and production kinetics of primebody hints that it may be produced under an atypical mechanism instead of the memory B cells. We reason that two possible sources for primebody can be supported from previous findings and from our data. One possible primebody source may be the immature B cells or B cells stimulated by various cytokines during class-switching. IgGs produced by B cells may lead to the production of IgGs with different glycan structures (6). However, this hypothesis can hardly explain the rapid rise-and-fall kinetics of primebody. The second hypothesis is based on the observation of the dynamic changes for different IgG glycoforms during the primebody production. In our MS glycoform profiling of total IgG from a KIN/CFA-immunized rabbit, we found that >20% of nonfucosylated, agalactosylated (G0) IgG disappeared at the same time the primebody was produced and exhibited a kinetics curve that was opposite of the primebody curve (Chen, unpublished data, 2011). Therefore, the primebody appeared to be a converted product of G0 IgG that disappeared. In other words, instead of freshly produced IgGs, primebody could be an IgG that was further glycosylation-modified from existing G0 IgGs during recycling between cells and the blood stream. Our other experiments suggest that the possible cells may include dendrite cells, macrophages, and endothelial cells that continuously take up and recycle serum IgGs (Chen, unpublished data, 2011). This hypothesis can also explain why the primebody disappeared within 3 to 5 days. More experiments are being conducted to provide more evidence for this “recycling and modification” hypothesis.

4. SUMMARY

We have systematically studied a unique IgG subset that was induced during humoral responses, but was not selective or specific to the immunogens. Our structure and functional analyses provide evidence that the primebody possesses a special Fc glycan structure and possible open conformation that is distinct from other serum IgGs. Because the generation of primebody is a common event for all animals and its detection is possible with our immunogen-independent assay, primebody can be used as a universal biomarker for an immune response to alert healthcare providers and first responders to possible infection, giving them a “curable window” for some fatal diseases, such as rabies, even without identification of the immunogen.

5. MATERIALS AND METHODS

5.1 Animal Care and Immunization

All animal studies were carried out in strict accordance with recommendations found in the *Guide for the Care and Use of Laboratory Animals* (U.S. Public Health Services and the National Institutes of Health, Washington, DC). Studies using BALB/c mice and white rabbits were conducted according to protocols approved by the Committee on the Ethics of Animal Experiments of Lampire Biological Laboratories, Inc. (LBL, Pipersville, PA). Animals were housed in LBL animal facilities, and all immunizations, blood draws, and serum preparations were performed according to LBL standard operating procedures. Studies using C57BL/6 mice were carried out in accordance with U.S. Public Health Service Policy on Humane Care and Use of Laboratory Animals under protocols approved by the Institutional Animal Care and Use Committee of the Thomas Jefferson University (TJU, Philadelphia, PA; Animal Welfare Assurance Number A3085-01). Mice were housed in TJU animal facilities, and all immunizations, blood draws, and serum preparations were performed according to TJU standard operating procedures.

As shown in Table 1, two strains of mice as well as NZW rabbits were used in this study. OVA, KIN (low molecular weight), and inactivated RV were used to inoculate animals through intraperitoneal or subcutaneous injection. Pre-immunization blood was drawn 7 days prior to immunization at LBL or on the day of immunization at TJU. Blood was drawn by either orbital bleeding (mice) or direct vein draw (rabbits) at different time points before and after immunization. The general frequency of blood draws was 3 to 5 days post-immunization during the first month, followed by immunogen booster injections, and then blood draws again at 7 to 10 day intervals after the first month. Pre-immunization blood was drawn 7 days prior to immunization at LBL and the day of immunization at TJU. Actual time points may vary by 1 to 2 days due to staff schedule changes and holidays.

5.2 Lectin and Antibodies

Wild-type AAL was purchased from Vector Laboratories, Inc. (Burlingame, CA). Recombinant AAL or α -1,6-fucose-specific mutants were constructed and expressed with a 10 residue histidine tag in *Escherichia coli*, and purified with nickel–nitrilotriacetic acid beads (Qiagen, Inc., Valencia, CA) (7). All antimouse or antirabbit IgG antibodies, including both whole IgG and F(ab')₂ fragments, were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA).

5.3 Antibody Microarray Preparation and Quantification of Primebody

High-density antibody microarrays were prepared for detecting antigen-specific IgG and AAL-reactive IgG, either on the same or on different microarrays, for different experiments in this study. For the selection of optimal capture antibodies for primebody detection, different antimouse or antirabbit antibodies, or F(ab')₂ fragments,

were printed onto each of the subarrays in a 1 × 3 in. ultrathin nitrocellulose slide (PATH Technology, Gentel Biosciences, Inc., Madison, WI) using a Scienion (Dortmund, Germany) FLEX ARRAYER S3 ultralow volume piezo microarrayer. About 300 pL of antibody sample was printed, resulting in high-density 130 μm diameter spots. The microarray contained 52 identical subarrays separated by wax grids formed with a wax imprinter (Gel Company, Inc., San Francisco, CA).

To detect primebody, slides were brought to room temperature and blocked with 1% IgG-free bovine serum albumen in 10 mM phosphate buffer solution (PBS, pH 7.2) with 0.5% Tween 20 (PBST, Sigma-Aldrich, St. Louis, MO) for 1 h at room temperature. After rinsing with PBST, 6 μL of serum samples diluted 100-fold were randomly applied onto the different subarrays to avoid prearrangement bias. Following three individual PBST rinses of 3 min each, biotinylated immunogen or lectin was applied onto each subarray to detect immunogen-specific and lectin-reactive IgGs. After a final rinse with PBST, the slide was probed with Dylight 549-labeled streptavidin (Thermo Fisher Scientific, Waltham, MA) and scanned by using a Perkin Elmer (Waltham, MA) Scan Array Lite microarray scanner at 10 μm. The images were analyzed and the data extracted by using the Genepix (Molecular Devices, Inc., Sunnyvale, CA) software package.

To quantify primebody, mouse or rabbit primebody standards were prepared with purified, denatured IgGs from mice or rabbits (Jackson ImmunoResearch Laboratories, Inc.) at known concentrations. Briefly, 1 mg/mL of purified IgG was incubated at 50 °C in PBS containing 20 mM dithiothreitol for 2 h and desalted with Biospin P-6 columns (Bio-Rad Laboratories, Hercules, CA) pre-equilibrated with PBS. The standards were serially diluted in PBS to concentrations between 0.001 and 100 μg/mL. The diluted samples were applied onto different subarrays of the microarray described previously, accompanied by other serum samples for primebody detection. The AAL-binding intensity of known standard and unknown serum samples were extracted as described above. Standard curves for primebody at different concentrations were plotted, and primebody concentrations in the unknown samples were determined from the primebody standard curves shown in Figures 1B and 1C. Goat F(ab')₂ antimouse IgG was used as the capture antibody for primebody detection in mice, and donkey F(ab')₂ antirabbit IgG was used for detection in rabbits.

5.4 Quantification of Immunogen-Specific and Total IgGs Using Antibody Microarrays

To determine the concentrations of immunogen-specific IgGs, a microarray containing 52 identical subarrays with immobilized immunogens and anti-IgG antibodies was used to capture immunogen-specific and total IgGs. Biotinylated, anti-IgG antibodies were then used for detection in an assay protocol similar to that described in Section 5.3.

5.5 Immunoprecipitation of Primebody and Immunogen-Specific IgG

Primebody was immunoprecipitated with recombinant AAL agarose beads (Pierce Biotechnology, Inc., now Thermo Scientific Pierce, Rockford, IL), and the immunoprecipitation of OVA-specific IgG was accomplished with OVA agarose beads. AAL and OVA agarose beads were prepared by incubating recombinant AAL and OVA protein, respectively, with N-hydroxysuccinimide-activated agarose beads according to the manufacturer's instructions. After washing with PBS (pH 7.2) containing 0.1% Tween 20, AAL or OVA agarose beads were added to rabbit serum samples and incubated overnight at 4 °C. Identical serum samples were also incubated overnight at 4 °C with empty agarose beads for use as a control. The bound proteins were eluted with 150 mM fucose or glycine hydrochloride (pH 3.0) for primebody or OVA-specific IgG, respectively. The OVA-specific IgG was immediately neutralized with 1 M tris(hydroxymethyl)-aminomethane base. The AAL immunoprecipitation eluent was further purified Melon Gels (Thermo Scientific, Inc.) according to the manufacturer's instructions.

5.6 Determination of Immunogen Binding Affinity for Purified IgG and Primebody Using Antibody Microarrays

To determine the antigen-binding affinity of purified IgG, antigen and antimouse or antirabbit F(ab')₂ were immobilized onto a protein-antibody microarray for the quantitation of bound IgG and total IgG simultaneously. Serial dilutions of purified immunogen-specific IgG were applied onto the microarray, and the amounts of immunogen-bound IgG were determined from the protein spots by following a similar procedure to that described in Section 5.5. The concentration of total IgGs was found by using a standard curved of serially diluted IgGs of known concentration. A curve was plotted using the bound IgG concentration as a function of total IgG concentration. EC₅₀ values were obtained as estimated K_d values for the immunogen-specific IgG binding to the immunogen. The procedure used to determine immunogen-binding affinity for primebody was identical to this purified IgG procedure.

5.7 IgG Fc N-Glycoform Profiling by MS

Quantification of IgG glycoisoforms by MS was carried out as described (8). Briefly, and as illustrated in Figure 7A, rabbit or mouse IgGs at different time points were purified from serum samples by Melon Gel according to the manufacturer's instructions. Purified IgGs were denatured and modified using dithiothreitol and 2-iodoacetamide, followed by trypsin digestion in ammonium bicarbonate buffer. Tryptic peptides were desalted using a homemade C₁₈ capillary column and redissolved in 2% acetonitrile and 0.1% formic acid.

LC-MS/MS analysis was performed using a QSTAR Elite quadrupole time-of-flight MS fitted with a Tempo nano LC system (Applied Biosystems, Inc.). Samples were loaded onto a precolumn (75 µm × 3 cm) packed with 5 µm Monitor C₁₈ particles (Column Engineering, Inc., Ontario, CA), then eluted with a linear 0 to 80% gradient of 98% acetonitrile and 0.1% formic acid over 80 min on a homemade analytical column

(75 μm $\times$ 10 cm, with 3 μm of Monitor C_{18} particles) with a 3 μm internal diameter tip and 2 kV potential difference. Precursor ions were scanned between 500 and 1800 m/z , and MS/MS spectra were acquired for selected ions under automatic collision energy. Ions for glycopeptides were also imported into the inclusion list for fragmentation using a fixed-collision energy ramp of 36 to 44 eV. Quantitative analysis of each glycosylation subtype was done in MS-only mode. The intensity of each subtype was obtained by adding the peak area of +2 and +3 forms of the same glycopeptide. For rabbit IgG, the glycopeptide sequence E₂₄₈QQFNSTIR₂₅₆ (GenBank number AAA64252.1) was used to define the molecular weight of the IgG for the identification and quantitation of the glycopeptide and its glycoforms. This is the only glycopeptide sequence found in the GenBank database (<http://www.ncbi.nlm.nih.gov/genbank/>).

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ACRONYMS AND ABBREVIATIONS

AAL	<i>Aleuria aurantia</i> lectin
CFA	complete Freund's adjuvant
C _H 2	(antibody) constant region heavy domain 2
EC ₅₀	50% effective concentration
ELISA	enzyme-linked immunosorbant assay
Fab	(antibody) antigen binding fragment
Fc	(antibody) constant region fragment
IFA	incomplete Freund's adjuvant
Ig	immunoglobulin
K _d	disassociation constant
KIN	kininogen
KLH	keyhole limpet hemocyanin
LBL	Lampire Biological Laboratories
LC	liquid chromatography
LC-MS/MS	liquid chromatography-tandem mass spectrometry
MS	mass spectrometry
OVA	ovalbumin
PBS	phosphate buffered saline
PBST	phosphate-buffered saline with 0.5% Tween 20
RV	rabies virus
TJU	Thomas Jefferson University
XIC	extracted ion chromatogram

